PI 3-kinase: a key effector in C-FMS signalling.

Thesis

UNRESTRICTED

PI 3-KINASE: A KEY EFFECTOR IN C-FMS SIGNALLING

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"for my grandmother"

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"ANYONE WHO HAS NEVER MADE A MISTAKE HAS NEVER TRIED ANYTHING NEW."

Albert Einstein (1879-1955)

Abstract

Abstract

Aberrant regulation of macrophage recruitment and activation by M-CSF is integral to inflammatory disease and cancer. M-CSF stimulated responses are mediated by the c-*fms* gene product found on monocytes and macrophages. A key component of signalling pathways immediately downstream of c-*fms* is Phosphatidylinositol 3-kinase. The research data presented in this thesis examined the role of PI 3-kinase in various M-CSF stimulated responses in a murine macrophage cell line, BAC1.2F5.

Analysis of the expression profile for PI 3-kinase identified p85α/p110α as the predominant PI 3-kinase isoforms present in BAC1.2F5 cells which was activated upon M-CSF stimulation. The PI 3-kinase specific inhibitors wortmannin and LY294002 were used to characterise the M-CSF stimulated responses in BAC1.2F5 cells. It was found that PI 3-kinase activity is required for protection of BAC1.2F5 macrophages against apoptosis, but not for the proliferation signal. It was observed that PI 3-kinase inhibitors prevented the correct transport of internalised c-*fms* from the plasma membrane to the endosomal/lysosomal compartments. In addition, PI 3-kinase inhibition also resulted in aberrant reorganisation of the actin cytoskeleton, characterised by the absence of fine actin cables within the cytoplasm of BAC1.2F5 cells.

In addition to the characterisation of c-*fms* signalling with respect to PI 3-kinase, this thesis also reports the production of human c-*fms* expression constructs containing mutations at critical PI 3-kinase binding sites and possessing C-terminal epitope tags for identification and purification . These constructs were expressed in HEK 293 cells and demonstrated to be recognised by both gene and epitope specific antibodies. Thus these constructs represent novel tools for the investigation of PI 3-kinase signalling.

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Abbreviations

Ab	Antibody
Ag	Antigen
AMP	Adenosine monophosphate
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BH	BCR-homology
BMM	Bone marrow derived macrophage
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
cDNA	complimentary DNA
°C	degree Celsius
Ci	Curie(s)
CIAP	Calf intestinal alkaline phosphatase
CMV	Cytomegalovirus
CPM	Counts per minute
DAG	Diacylglycerol
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylenediamine tetra-acetic acid
EGF	Epidermal growth factor
FACS	Fluorescence activated cell sorting
F-Actin	Filamentous actin
FCS	Foetal calf serum
FITC	Fluorscein isothyocyanate
GAP	GTPase activating protein
GTPase	Guanosine triphosphatase
Grb2	Growth factor receptor binding protein-2
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
G-CSF	Granulocyte-colony stimulating factor
GM-CSF	Granulocyte/macrophage-colony stimulating factor
GST	Glutathione S-transferase
Hepes	*N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulphonic acid
HRP	Horseradish peroxidase
IFN$_\gamma$	Interferon gamma
Ig	Immunoglobulin
IGF-1	Insulin-like growth factor-1
IL-χ	Interleukin-χ
IMS	Industrial methylated spirits
IP$_3$	Inositol trisphosphate
IPTG	Isopropyl thiogalactoside
IRS-1	Insulin receptor substrate-1
kb	kilobase
kbp	kilobase pair
kDa	Kilodalton
LB	Luria Bertani
LDL	Low density lipoprotein

LPA	Lysophosphatidic acid
LPS	Lipopolysaccharide
mAb	monoclonal antibody
MAPK	Mitogen activated protein kinase
M-CSF	Macrophage-colony stimulating factor
M_r	relative molecular mass
mRNA	messenger RNA
NRTK	Non-receptor tyrosine kinase
Op	Osteopetrotic
P	Probability
p	protein
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PDK-1	PIP_3-dependent protein kinase-1
PH	Pleckstrin homology
PI	Phosphatidylinositol
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
pp	phosphoprotein
PTB	Phosphotyrosine binding
PTK	Protein tyrosine kinase
PTP	Protein tyrosine phosphatase
RNA	Ribonucleic acid
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription-polymerase chain reaction
SAPK	Stress activated protein kinase
SDS	Sodium dodecyl-sulphate
SDM	Site directed mutagenesis
SEM	Standard error of the mean
SH2	Src homology-2
SH3	Src homology-3
SHIP	SH2 domain containing inositol phosphatase
SHP-1	SH2 domain containing phosphatase-1
Sos1	Son of sevenless 1
STAT	Signal transducers and activators of transcription
TEMED	Tetramethylethylenediamine
TGN	*Trans*-golgi network
TLC	Thin layer chromatography
TNF	Tumour necrosis factor
TRITC	Tetramethylrhodamine isothiocyanate
Vps	Vacuolar protein sorting
X-Gal	σ-Nitrophenyl-β-D-galactopyranoside

Chapter 1

Introduction

1.1.0. Macrophages in the immune system

1.1.1. The immune system

In humans and other mammals the immune system facilitates defence against pathogens and infectious agents such as bacteria, viruses, fungi, protozoa as well as multicellular parasites. The innate and adaptive immune systems not only protect us from these potentially infectious agents but also from ourselves during episodes of potentially uncontrolled growth such as malignancy or autoimmune diseases (Clancey, 1998). In order to combat certain pathogens, multicellular organisms have developed a group of cells which circulate through the bloodstream, lymphatic system and infiltrate tissues which possess specific receptors that recognise immunogenic peptide fragments of both intracellular and extracellular pathogens. When these receptors are triggered, the immune cells secrete various soluble mediators such as antibodies or perforin granules which attack pathogens directly or stimulate other cells to do so via the production of cytokines. This circulating army of cells includes B and T lymphocytes and various antigen presenting cells like monocytes, macrophages and dendritic cells such as basophils, eosinophils, neutrophils and mast cells (Clemens, 1991).

1.1.2. Macrophages and immunity

The macrophage is an extremely important mediator of the immune response. Macrophages develop and mature from bone marrow precursors before entering the bloodstream as monocytes (Clemens, 1991). Monocytes are recruited into different tissues where they further differentiate into resident tissue macrophages which perform important functions in tissue homeostasis and the immune response (Lewis, 1992). Macrophages are found in all tissues and primarily act as scavengers during

the innate immune response, phagocytosing large particles such as bacteria, yeast and dying cells (Lewis, 1992). The macrophage, although a very competent phagocyte during innate immunity, also plays an important role as an antigen presenting cell and as an effector cell during cell-mediated immunity (Lewis, 1992). Macrophages also process foreign antigens and present them to T-lymphocytes allowing development of a specific immune response. In addition macrophages produce chemokines and cytokines, signalling molecules that orchestrate the immune response and stimulate the secretion of proteases and growth factors, which are important for tissue remodelling and wound repair after injury (Lewis, 1992). Thus macrophages respond to a wide variety of inflammatory mediators and play a central role in acute and chronic inflammation (Lewis, 1992).

1.1.3. Macrophage phagocytosis and vesiculation.

Macrophages phagocytose small particulate matter including small latex beads and large polymer beads, up to twice the diameter of the cell, which are ingested by surrounding the particle with extrusions from the cell membrane. Although phagocytosis usually results in the destruction of the ingested particle by production of reactive oxygen or nitrogen metabolites or by proteolysis, some pathogens, including the *mycobacteria* which cause tuberculosis or leprosy, evade immune destruction by surviving inside the macrophage which is an important feature of their pathogenesis. In general, most cells in culture take up small volumes of culture medium by a process called pinocytosis (Swanson and Watts, 1995). However macrophages constitutively take-up large volumes of external fluid via macropinocytotic vesicles (Swanson and Watts, 1995). Macrophages can also be stimulated to form macropinocytotic vesicles by growth factors and cytokines such as IL-6, GM-CSF or M-CSF (Araki *et al.*, 1996; Swanson and Watts, 1995). The role of

macropinocytosis is still unclear but various benefits to macrophages include routine turnover of plasma membrane proteins; a mechanism for re-routing membrane traffic; and importantly the non-specific capture of antigens prior to expression of high affinity antibodies (Swanson and Watts, 1995).

1.1.4. Cytokines secreted by macrophages

Macrophages can be stimulated by various agonists to secrete tumour necrosis factor alpha (TNFα), interleukin (IL) -1β, IL-6, IL-8, IL-10 and IL-12 (Lewis, 1992). The binding of TNFα to its receptor on macrophages initiates the production of various cytokines which mediate the inflammatory response and induces the expression of many genes important for the host response to infection (Lewis, 1992). IL-1β and IL-6 are pro-inflammatory cytokines that act either in an autocrine manner or systemically to elicit production and recruitment of neutrophils, the synthesis of acute phase proteins by the liver and the activation of T-cells (Lewis, 1992). IL-8 is also produced by macrophages and is the main cytokine involved in neutrophil recruitment to sites of tissue damage or infection (Clemens, 1991). Not only is IL-10 a potent modulator of monocyte and macrophage function, involved in the deactivation of macrophages, but it also suppresses IL-2 and interferon gamma (IFNγ) production by Th1 T-cells. Production of IL-12 stimulates the growth of activated natural killer (NK) cells, $CD8^+$ and $CD4^+$ T-cells (Lewis, 1992).

1.1.5. Cytokines and growth factors that activate macrophages

Macrophage activation and function is regulated by an array of proteins and small molecules including IFNγ, IL-4, IL-13, TGFβ and the colony stimulating factors (CSFs). IFNγ, produced by Th1 T-cells and NK cells, activates macrophage pathogenicity and also upregulates expression of the macrophage major

histocompatability complex (MHC) class I, IL-1, platelet activating factor (PAF) and H_2O_2 production (Lewis, 1992). In addition, IFNγ can act in concert with lipopolysaccharide (LPS) to upregulate nitric oxide production (Lewis, 1992). IL-4 and IL-13 have similar biological effects and stimulate macrophage fusion in culture to form multi-nucleate giant cells (Lewis, 1992). TGFβ stimulates macrophage secretion of various growth factors including platelet derived growth factor beta (PDGFβ) and extracellular matrix (ECM) components (Lewis, 1992). TGFβ has both positive and negative effects on macrophages including stimulating chemotaxis and the secretion of tissue inhibitors of metalloproteinases (TIMPs) and inhibition of nitric oxide synthesis (Lewis, 1992).

The colony stimulating factors are a family of haematopoietic cell growth factors that stimulate the growth and differentiation of cells of the granulocyte and macrophage cell lineages (Das and Stanley, 1982). The macrophage colony stimulating factor (M-CSF or CSF-1) is produced by many cells including macrophages and is important for the survival, proliferation and differentiation of monocytes, macrophages, osteoclasts and their precursors (Stanley *et al.*, 1997; Stanley *et al.*, 1976). M-CSF also stimulates macrophages to produce plasminogen activator, prostaglandin E and also enhances both phagocytic and tumoricidal activities (Clemens, 1991). The role of M-CSF and its receptor in macrophage function will be discussed later in greater detail.

1.1.6. Macrophages and disease

One of the most common causes of death in the western world is cardiovascular disease. Atherosclerosis, the principal cause of myocardial and cerebral infarction, accounts for the majority of these deaths. Monocyte recruitment is a key event in the formation of the earliest vascular lesions, the fatty streak in atherosclerotic plaques

(Campbell and Campbell, 1997; Lewis, 1992). It has been shown that uptake of modified low-density lipoproteins (LDLs) by monocyte-derived macrophages gives rise to the foam cells present in the atheroma which accumulate in the latter stages of plaque formation and release lipids into the intima of the blood vessel (Campbell and Campbell, 1997). Vascular smooth muscle cells in the plaque proliferate in response to growth factors such as PDGFβ released by macrophages and this leads to plaque fibrosis (Lewis, 1992). In addition, macrophages secrete other chemotactic cytokines (chemokines) which lead to further macrophage recruitment, infiltration and the onset of chronic inflammation (Lewis, 1992). A model of atherosclerosis can be created either by feeding mice a diet containing high fat and cholesterol (Qiao *et al.*, 1997) or by crossing the mouse knockout for M-CSF (op/op), which will be discussed later in greater detail, with the apolipoprotein E (apo E) knockout mouse (Qiao *et al.*, 1997). In both the dietary and apo E knockout models of atherogenesis, the M-CSF knockout resulted in significantly reduced atherogenesis (Qiao *et al.*, 1997) and in LDL receptor-deficient mice both heterozygous (+/-) and homozygous osteopetrotic phenotypes (-/-) reduced atherosclerosis (Rajavashisth *et al.*, 1998). Therefore M-CSF regulates recruitment of circulating monocytes and macrophages to the atherosclerotic lesion and their contribution to atherogenesis.

Aberrant macrophage function is also implicated in other disease states including rheumatoid arthritis (RA). Macrophage activation and recruitment in synovial tissue participate in the development of RA lesions and mediate of cartilage and bone destruction (Amento *et al.*, 1982). The use of anti-TNF antibodies or soluble TNF receptor fusion proteins to reduce circulating and localised TNF levels within joints dramatically decreases the severity of the disease (Arend and Dayer, 1995; Williams

et al., 1992). Therefore disruption of aberrant monocyte/macrophage recruitment to RA joints is therapeutically beneficial.

Monocytes and macrophages also possess tumouricidal, cytocidal and microbicidal capacity. As a consequence of their broad range of functions macrophage dysfunction is also involved in a wide variety of immunodeficient conditions, including common variable immunodefficiency (CVID) and chronic granulomatous disease (CGD), although this is predominantly a neutrophil deficiency disease (Donowitz and Mandell, 1982; Eibl *et al.*, 1982).

Abnormal monocyte and macrophage function has been detected in many human cancers. During the progression of Hodgkin's disease there is increased macrophage expression of Fc receptor, phagocytosis and an increase in peripheral blood monocyte population (Sciborski, 1989). Macrophages normally respond chemotactically to growth factors, but this is reduced in lung and prostate cancers, renal cell carcinoma and melanomas (Lewis, 1992). Enhancement of macrophage tumouricidal activity represents a valuable area for therapeutic intervention. Augmentation of monocyte and macrophage cytostatic and cytotoxic activity towards tumour cells is currently being extensively investigated in many laboratories. To date IFNα and GM-CSF have shown positive therapeutic benefits in a number of cancer patients (Borden and Sondel, 1990; Fuith *et al.*, 1989; Steward *et al.*, 1989).

1.2.0. Biology and action of Macrophage-Colony Stimulating Factor

1.2.1. Macrophage-Colony Stimulating Factor

M-CSF was originally identified by Stanley and colleagues as a potent growth factor activity present in L-cell conditioned medium that promoted the survival and proliferation of macrophages *in vitro*, and stimulated the formation of macrophage colonies in soft agar (Sherr, 1988; Stanley *et al.*, 1976). M-CSF is a disulphide-linked homodimeric glycoprotein produced by a number of cell types, principally monocytes and macrophages, but also fibroblasts, epithelial and endothelial cells, activated B- and T-cells and some tumour cell lines (Sherr, 1988; Sherr *et al.*, 1985). M-CSF is also secreted by a number of established cell lines *in vitro* including the L-929 murine fibroblast-like line (L-cell) (Stanley *et al.*, 1976). Synthesis of the growth factor by macrophages is stimulated by the individual or collective presence of IL-1, PDGF, granulocyte-macrophage colony stimulating factor (GM-CSF), γ-interferon (γ-IFN) and tumour necrosis factor-α (TNF-α) (Sherr, 1988). In addition the synthesis of M-CSF is negatively regulated by prostaglandins, glucocortcoids and TGFβ (Blake *et al.*, 1998).

M-CSF stimulated responses include; enhancement of antibody dependent cell-mediated cytotoxicity by monocytes and macrophages, inhibition of bone resorption by osteoclasts, stimulation of microglial cell proliferation and the regulation of placental function via the action of decidual cells and trophoblasts (Hattersley *et al.*, 1988; Mufson *et al.*, 1989; Welte *et al.*, 1985). Synergism of M-CSF with IL-1, IL-3 and IL-6 stimulates primitive haematopoietic cells to proliferate and differentiate into mature macrophages (see Fig. 1.2.1.) (Ibelgauft, 1998). M-CSF is also required by tissues that are undergoing rapid morphogenesis or tissue remodelling (Stanley *et al.*, 1997).

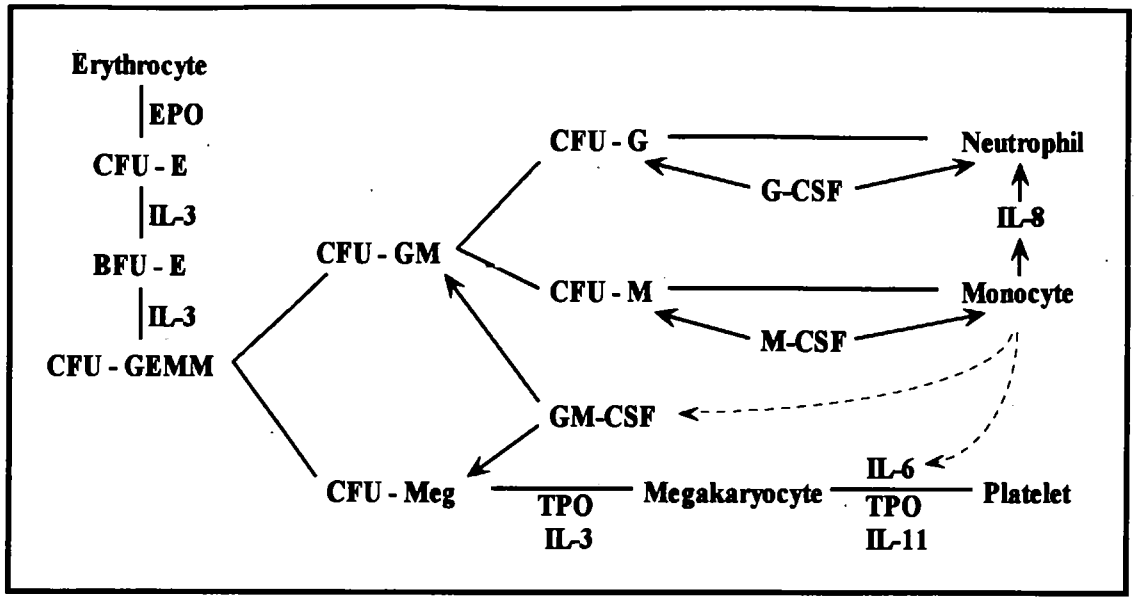

Fig. 1.2.1. M-CSF, a growth factor that acts during haematopoiesis.

Secreted human and murine M-CSF are highly homologous at the amino acid level (>80%), and although human M-CSF exhibits cross species activity with the murine receptor the murine M-CSF does not act on the human receptor (Das and Stanley, 1982). Indeed, it appears that human M-CSF is a more potent stimulator of murine macrophage activity *in vitro* (Das and Stanley, 1982). M-CSF binds with high affinity ($K_d = 0.4x10^{-10}$ for human M-CSF and $K_d = \approx 2x10^{-10}$ for murine M-CSF) to its specific cell-surface receptor, which is found on virtually all mononuclear phagocytes (Das and Stanley, 1982; Welte *et al.*, 1985). In M-CSF deprived, phagocytic cells, cell-cycle arrest occurs with prolonged starvation leading to apoptosis (Li and Stanley, 1991). Re-introduction of M-CSF, following short periods of deprivation (12-18 hrs) induces re-entry into the cell-cycle with concomitant expression of immediate early gene expression including *fos, myc* and *jun* (Li and Stanley, 1991). The transcription of delayed early genes then follows, which includes the D-type cyclins and their catalytic partners which are crucial for G_1 progression and the initiation of DNA synthesis (Sherr, 1993). A continual requirement for M-CSF from initiation of immediate early gene expression through G_1 has been

demonstrated, and therefore it is believed that M-CSF acts as a progression factor as well as a competence factor (Sherr, 1993).

Human M-CSF is encoded by a single gene composed of 10 exons located on human chromosome 1p21-1p13, (chromosome 3 in mice) (Gisselbrecht et al., 1989; Yaar, 1998). Molecular cloning data has determined that two alternatively spliced mRNA species exist for the human M-CSF which code for three M-CSF species (Hampe *et al.*, 1989; Ibelgauft, 1998). The smaller transcript of 1.8-2.0kb lacks the 5'-end of exon 6, and codes for a 36-52kDa membrane bound, disulphide-linked homodimer (Ibelgauft, 1998; Roussel, 1994). The larger, 4.0kb mRNA species containing the 5' end of exon 6, codes for a secreted 70-90kDa disulphide linked homodimer (Welte *et al.*, 1985). The 4.0kb transcript is also processed as a proteoglycan, soluble, secreted form with an apparent molecular weight of 150-200kDa (Ibelgauft, 1998). Both secreted and membrane bound M-CSF play diverse physiological roles in stimulation of receptor-bearing cells, by either endocrine or paracrine mechanisms or through direct cell-cell contact (Sherr and Stanley, 1990). The secreted proteoglycan form of M-CSF may also be sequestered to specific locations by virtue of its glycosaminoglycan chains (Stanley *et al.*, 1997). Deletion mapping experiments have demonstrated that the first 149 amino acids of human M-CSF are minimally required for biological activity (Clemens, 1991).

1.2.2. Clinical relevance of M-CSF

In the past 15 years the application of growth factor therapy to a variety of diseases has been explored by several laboratories and clinics. At present a number of clinical trials are being conducted using Granulocyte-CSF (G-CSF) and Granulocyte/ Macrophage-CSF (GM-CSF) as treatments for various diseases including

chemotherapy-induced neutropenia in cancer patients (Nemunaitis, 1997). Both GM-CSF and G-CSF have been approved by the United States food and drug administration (US FDA) for clinical trials (Nemunaitis, 1997). The Japanese FDA have also approved recombinant M-CSF for therapeutic use in acceleration of granulocyte recovery following allogenic transplant, dose-intensive therapy of ovarian cancer and induction therapy of acute myelogenous leukaemia (AML) (Nemunaitis, 1997). M-CSF has also been evaluated as an anti-microbial agent since in mice it promotes survival of pathogen-infected mice (Nemunaitis, 1998). Clinical trials on M-CSF have also been conducted in patients with fungal infection following bone marrow transplantation (BMT). Co-administration of amphotericin B with M-CSF enhanced patient survival in pathogen-infected patients compared to patients treated with only amphotericin B (Nemunaitis, 1997). Elevated M-CSF levels have also been reported in malaria infection which results in macrophage activation and leads to the increased macrophage-mediated platelet destruction associated with disease progression (Lee *et al.*, 1997).

1.2.3. M-CSF signalling and carcinogenesis

Increased levels of circulating M-CSF have been associated in myeloproliferative diseases including myeloid metaplasia, peripheral bone marrow extension (Gilbert *et al.*, 1989) and in an experimental mouse model of AML (Haran-Ghera *et al.*, 1997). Increased levels of circulating M-CSF have also been detected in myelodysplastic syndrome (MDS), chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), non-Hodgkin's lymphoma (NHL), Hodgkin's disease (HD), and multiple myeloma (MM) (Janowska-Wieczorek *et al.*, 1991). Taken together, it has become apparent that M-CSF is an important factor for tumour progression, particularly in tumours of the haematopoietic lineage, and may play a regulatory role

in the maintainence of these disease states. In addition a number of different tumour types also express the M-CSF receptor, c-*fms*, which suggests that M-CSF regulation of tumour progression may be controlled, at least in part, through an autocrine loop.

Elevated levels of M-CSF may potentially be a more accurate indicator of the presence and activity of either primary endometrial adenocarcinoma or recurrent disease than the established marker, CA 125 (Hakala *et al.*, 1995; Kacinski *et al.*, 1989). Circulating levels of M-CSF have also been investigated as a potential indicator of the response to chemotherapy in acute type adult T-cell leukaemic (ATL) patients (Yamada *et al.*, 1996). Circulating M-CSF levels declined when patients achieved complete or partial remission but increased again when partial remission patients showed renewed disease progression (Yamada *et al.*, 1996). Gene transfer trials of human M-CSF expression in tumour transplantation models have shown that when the human M-CSF gene is expressed in J558 plasmacytoma cells it does not suppress tumour growth, but expression in B16 melanoma cells and 3LL lung cancer cells induced immunity against the parent tumour cells (Motoyoshi, 1998). Therefore it appears that therapeutically, M-CSF can also be used to treat certain types of cancer in addition to chemotherapy side affects such as neutropenia, but its use is limited to the type of disease being treated.

1.2.4. The M-CSF knockout mouse: osteopetrotic (op/op)

The mouse knock-out for M-CSF, (osteopetrotic or op/op) is a classical mutation with a homozygous genotype caused by a null allele for M-CSF expression (Wiktor-Jedrzejczak *et al.*, 1990). Analysis of cDNA isolated from op/op fibroblasts suggests that a single base insertion causes a frame-shift mutation in the coding region of the M-CSF gene introducing a stop codon which gives rise to synthesis of a non-

functional polypeptide (Pollard, 1997). Homozygous op/op mice have a viable phenotype, but exhibit severe macrophage deficiency resulting in a reduced haematopoietic activity, neurological defects and an absence of a functional osteoclast population (Yoshida *et al.*, 1990). However, by about forty weeks the symptoms of M-CSF deficiency appear to be corrected. This apparent partial reversal of phenotype is thought to occur through an adaptation to the absence of M-CSF by other growth factors which also stimulate macrophage development (Yoshida *et al.*, 1990).

Op/op mice do not respond normally to external stimuli and the phenotype is characterised by cortical processing problems including excitatory and inhibitory processing problems in nerve signals from the retina along the optic nerve (Cecchini *et al.*, 1994; Pollard, 1997). However, there are no apparent morphological differences between the brains of op/op mice or their normal littermates, but in normal mouse development a unique mRNA splice variant encoding a secreted form of M-CSF found only in brain tissue is absent in op/op mice (Cecchini *et al.*, 1994). The op/op phenotype, including the observed developmental abnormalities, can be rescued by daily injection of recombinant M-CSF (Cecchini *et al.*, 1994). The age-related recovery of haematopoietic function and resolution of osteopetrosis observed in the op/op phenotype also seems to indicate that the developing haematopoietic system has the capacity to use alternative mechanisms to M-CSF signalling and can compensate for its absence. Interestingly, there is an elevated level of splenic haematopoietic activity during the period of time in which the immune system develops and coincides with the period of reduced haematopoietic activity in the bone marrow of developing op/op mice (Begg *et al.*, 1993). Op/op mice are also capable of mounting an apparently normal T-cell-dependent immune response (Chang *et al.*, 1995). In addition, op/op mice are able to induce Ag-specific proliferation of naïve T-cells,

generate cytotoxic T-lymphocytes, supply spleen cells to serve as stimulators in a mixed lymphocyte reaction and produce IgM and IgG, similar to their normal littermates (Chang *et al.*, 1995). However, M-CSF-deficient mice do not develop autoimmune haemolytic anaemia on injection with anti-red blood cell monoclonal antibodies or autoimmune thrombocytopaenia on injection of anti-platelet monoclonal antibodies (Clynes and Ravetch, 1995) which is probably due to an absence of an Fc gamma receptor bearing, circulating macrophage population which normally regulates opsonised phagocytosis of red blood cells and platelets (Begg *et al.*, 1993; Michaelson *et al.*, 1996; Pollard and Hennighausen, 1994; Stanley *et al.*, 1997; Wiktor-Jedrzejczak *et al.*, 1990). Therefore it may be concluded that macrophage populations which are either M-CSF independent or have adapted to the absence of M-CSF have developed in the op/op phenotype and they can participate in a limited number of macrophage functions but the full macrophage repertoire remains unavailable in the op/op mouse.

1.2.5. Requirement for M-CSF during pregnancy

During pregnancy M-CSF concentrations in the mouse uterus are elevated up to a thousand fold, whereas serum levels remain relatively normal (Pollard, 1997). In situ hybridisation has shown that all four M-CSF transcripts are expressed but only in the uterine luminal and glandular epithelium (Pollard, 1997). However, in the op/op mouse female absence of M-CSF in the uterine epithelium correlates with lower fertility compared to their normal littermates, and is characterised by defective folliculogenesis and ovulation, where the number of ovulated ova is significantly smaller compared to normal females (Stanley *et al.*, 1997). M-CSF participation in folliculogenesis and ovulation appears to be indirect, controlling the numbers of ovarian macrophages present, but it may have a direct effect on granulosa cells and

oocytes (Stanley *et al.*, 1997). Rescue of the op/op placental phenotype can be achieved by M-CSF therapy and also by opportunistic stimulation of c-*fms* by steel factor, a structural relative of M-CSF (Stanley *et al.*, 1997).

Macrophages are also found throughout the male reproductive tract and its accessory glands (Pollard *et al.*, 1997). Interestingly the op/op males exhibit reduced libido compared to normal males as well as a significantly lower density of macrophages in the testis, epididymis, prostate, seminal vesicles and vas deferens (Pollard *et al.*, 1997). This suggests that occurrence of macrophages is regulated by M-CSF in male reproductive tissues and addition of M-CSF restored the numbers of resident macrophages to that normally present in all tissues except the prostate (Pollard *et al.*, 1997). The male op/op phenotype also shows a seven fold decrease in the concentration of circulating testosterone with significantly reduced intratesticular testosterone levels compared to their normal littermates (Cohen *et al.*, 1996). Reduced testosterone levels are consistent with the reduction in mating capability and reduced sperm viability observed in op/op males and this phenotype can be rescued by treatment with either recombinant testosterone or M-CSF, which suggests that M-CSF regulates testosterone levels as well as the presence of testicular macrophages (Cohen *et al.*, 1996).

1.2.6. M-CSF during development

Through studies in normal and op/op mice it has become apparent that M-CSF expression is developmentally regulated and is required for tissue morphogenesis. Table 1.2.6. is modified from Cecchini *et al*, 1994 and shows a summary of the requirement for M-CSF during tissue morphogenesis in the developing mouse (Cecchini *et al.*, 1994). The first three months of postnatal development in op/op

mice and their normal (+/op or +/+) littermates were monitored for expression of tissue F4/80-positive (F4/80+) cells, a macrophage cell marker antigen, which generally correlates with organogenesis and/or cell turnover. Depending on the tissue, the F4/80+ cell density either decreases, transiently increases or gradually increases with age and in op/op mice, tissues that normally contain F4/80+ cells were classified into those in which F4/80+ cells were absent and those in which the F4/80+ cell densities were either reduced, normal or initially normal then subsequently reduced. Macrophages of the dermis, bladder, bone marrow and salivary gland, together with a subpopulation in the gut can be partially restored by subcutaneous injection of recombinant M-CSF which restored circulating M-CSF levels (Cecchini *et al.*, 1994). However, macrophages found in muscle, tendon, periosteum, synovial membrane, adrenals and the epithelia of the digestive tract, are not corrected by restoration of circulating M-CSF, which suggests that they have an absolute requirement for and are locally regulated by M-CSF (Cecchini *et al.*, 1994).

Important differences exist among macrophages in their dependency on M-CSF and the manner of M-CSF presentation. M-CSF influences organogenesis and tissue turnover by stimulating the production of tissue macrophages with local trophic and/or scavenger functions (Cecchini *et al.*, 1994). In addition Roth *et al*, 1996 have characterised the levels of M-CSF protein and mRNA expression in foetal, neonatal and maternal mice (Roth and Stanley, 1996). M-CSF levels increase in liver, kidney and lung tissue but decrease in brain and serum with increasing age (Roth and Stanley, 1996). However, embryonic M-CSF concentrations are higher in liver, kidney and serum but lower in lung, brain, intestine and heart compared to adult mice (Roth and Stanley, 1996).

Tissue	M-CSF Requirement	Pre-natal	Post-natal	Response to post-natal circulating M-CSF
Muscle	**Absolute**	+	-	**None**
Tendon	**Absolute**	+	-	**None**
Dermis	**Absolute**	+	-	**Partial/none**
Periostium	**Absolute**	+	+ (early)	**None**
Synovium	**Absolute**	+	+ (early)	**None**
Kidney	**Absolute**	+	+ (early)	**Complete**
Retina	**Absolute**	+	+ (early)	**Complete**
Adrenals	**Partial**	-	+	**None**
Bladder	**Partial**	-	+	**Partial/none**
Salivary gland	**Partial**	-	+	**Partial**
BM macrophage	**Partial**	+	+	**Partial**
Liver	**Partial**	+	+	**Complete**
Stomach	**Partial**	-	+	**Partial/none**
Gut	**Partial**	-	+	**Partial/none**
Spleen	**Partial**	+	+	**Complete**
Epidermis	**None**	?	-	**None**
Thymus	**None**	?	-	**None**
Lymphnode	**None**	-	-	**None**
BM monocyte	**None**	-	-	**None**

Table 1.2.6. Summary of the effects of M-CSF on the regulation of tissue macrophage development. Modified from Cecchini *et al.*, 1994.

During development, M-CSF expression is detected in the embryonic whole brain, except in the hippocampus and striatum (day 16) while c-*fms* transcripts are detectable in microglial cells and in cells of the hippocampus, striatum, cerebellum and cortex (day 16) (Pollard, 1997). Expression of the unique neuronal M-CSF splice variant may regulate cell growth, proliferation or differentiation of neuronal cells that express c-*fms* (Pollard, 1997) and it has been demonstrated that M-CSF stimulates survival and neurite outgrowth in neurons from embryonic brain explants in a dose dependent manner (Michaelson *et al.*, 1996).

1.3.0. The receptor for M-CSF

1.3.1. Protein tyrosine kinases

Protein tyrosine kinases (PTKs) are enzymes that catalyse the transfer of the γ-phosphate of adenosine trisphosphate (ATP) to tyrosine residues of protein substrates (Hubbard *et al.*, 1998). PTKs are critical components of signalling pathways that control cell proliferation, differentiation, locomotion and other specific cell functions in response to the extracellular environment (Hubbard *et al.*, 1998). PTK activities can be subdivided into two large families; receptor tyrosine kinases (RTKs) and non-receptor tyrosine kinases (NRTKs). NRTKs possess no extracellular or trans-membrane domains but contain modular domains responsible for subcellular targeting and regulation of catalytic activity (Hubbard *et al.*, 1998). RTKs however, possess extracellular domains that facilitate interaction with the external environment and are broadly divided into two groups, depending on their covalent organisation (Hubbard *et al.*, 1998). Most RTKs are a single polypeptide chain which remains monomeric in the absence of ligand. Ligand binding to the extracellular portion of the receptor leads to dimerisation of the monomeric receptor or rearrangement within the quaternary structure of heterotetrameric receptors, which results in autophosphorylation of specific tyrosine residues in the cytoplasmic portion of the receptor (Hubbard *et al.*, 1998). In general, tyrosine phosphorylation stimulates intrinsic receptor kinase activity generating recruitment sites for downstream signalling proteins containing phosphotyrosyl motif recognition domains such as *src* homology 2 (SH2) or phosphotyrosine binding (PTB) domains (Hubbard *et al.*, 1998). Therefore each cell contains mechanisms by which changes in the extracellular environment can be recognised by specific cell surface proteins which then become phosphorylated within their cytoplasmic regions, thus allowing intracellular proteins

to associate and further transduce a signal. Binding of dimeric ligands such as M-CSF or PDGF to their receptors induces a symmetric receptor dimerisation within the extracellular domains and leads to RTK autophosphorylation and activation of kinase activity (Fig. 1.3.1.). The ligand-induced activation of the receptor for M-CSF, c-*fms* will be discussed below in greater detail.

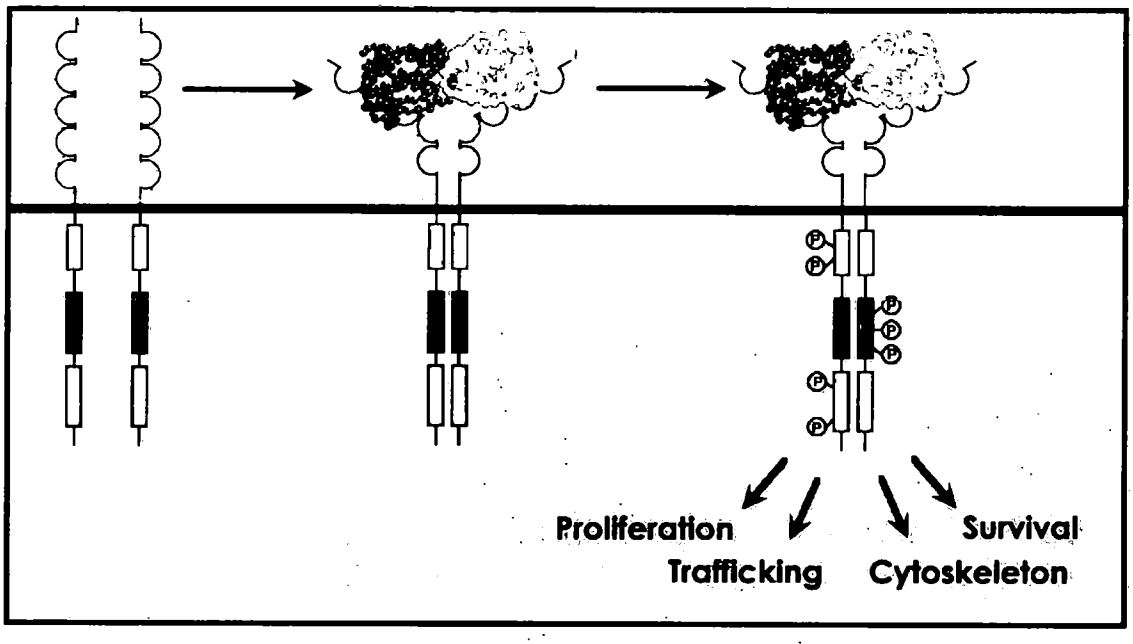

Fig. 1.3.1. Ligand-binding and receptor autophosphorylation by dimeric receptor tyrosine kinases.

1.3.2. The M-CSF receptor: c-*fms*

The cell surface receptor for M-CSF is the protein product of the c-*fms* proto-oncogene, and is expressed in blood, spleen, liver, brain and bone marrow of adult animals, reflecting the distribution of monocytes and macrophages (Rettenmier *et al.*, 1988; Sherr *et al.*, 1985). C-*fms* expression has been detected in breast carcinoma tissue, smooth muscle cells from atherosclerotic lesions, B-lymphocytes and transcripts have been detected in placental trophoblasts and uncharacterised embryonic cells suggesting that c-*fms* signalling is also required outside haematopoiesis (Baker et al., 1995; Inaba et al., 1992; Kacinski et al., 1991; Rettenmier et al., 1988; Sherr, 1988).

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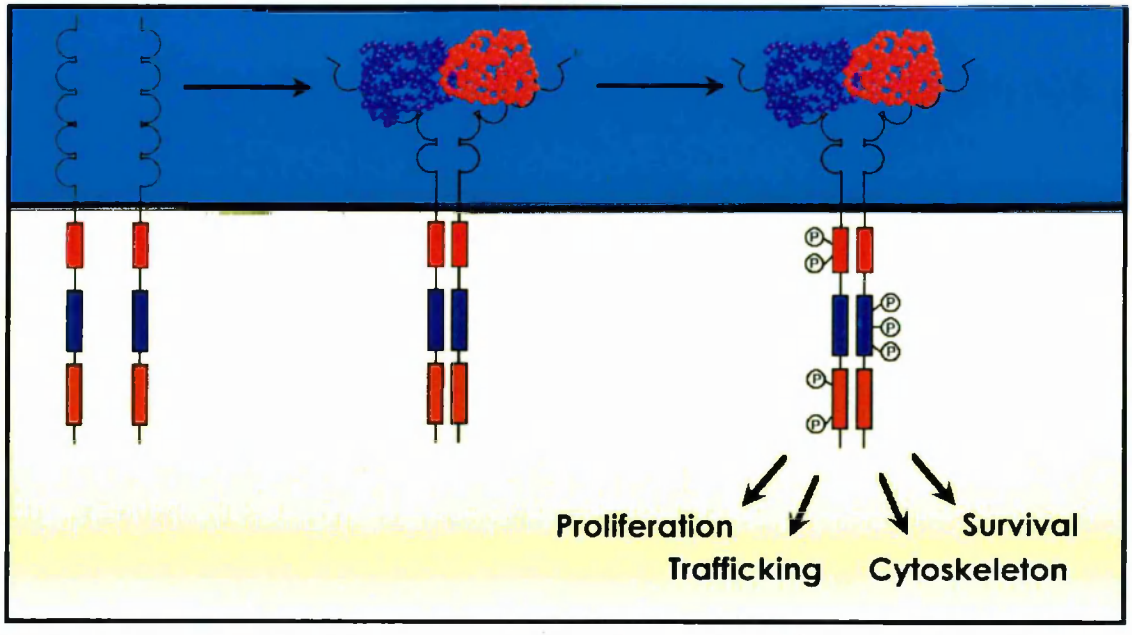

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The c-*fms* protein product is a transmembrane glycoprotein composed of an extracellular ligand-binding domain and a cytoplasmic domain that possesses an intrinsic tyrosine kinase activity (Sherr *et al.*, 1985). Binding of M-CSF to c-*fms* results in receptor auto-phosphorylation followed by rapid internalisation and degradation of the ligand-receptor complex (Ohtsuka *et al.*, 1990; Roussel, 1994; Tapley *et al.*, 1990). Ligand binding causes immediate changes in the membrane structure of macrophages, including the formation of filopodia, vesiculation and enhancement of phagocytic activity (Boocock *et al.*, 1989; Rettenmier *et al.*, 1988). M-CSF binding to c-*fms* on macrophages also results in the rapid increase in intracellular pH levels and secondary stimulation of Na^{+}/K^{+} ATPase activity, via activation of the Na^{+}/K^{+} antiporter (Sherr, 1988). Auto-phosphorylation occurs at specific tyrosine residues on c-*fms* and represents the initial event in receptor activation and hence signal-transduction (Tapley *et al.*, 1990). It has been demonstrated that PI 3-kinase and various other effector proteins bind to the activated c-*fms* (Sherr, 1990; Varticovski *et al.*, 1989). C-*fms* signalling has not been investigated as extensively as other haematopoietic growth factor receptors, however subtle differences have been observed in c-*fms* signalling that distinguish it from other RTK family members (Rettenmier *et al.*, 1988; Roussel, 1994).

1.3.3. The genomic location of c-*fms*

Multiple alleles exist for human c-*fms*, which is located on chromosome 5q33.3-34 approximately 1kbp downstream from the PDGFr (Yaar, 1998). The close proximity between PDGFr and c-*fms* on chromosome 5 suggests expression of both these receptors may be controlled by common regulatory sequences, since c-*fms* appears to have no regulatory sequences of its own possibly arising from gene duplication (Blake *et al.*, 1998). In other species, c-*fms* is localised to chromosome 7 in cattle,

chromosome 5 in dogs, chromosome 2 in chinese hamsters, chromosome 3 in Owl monkeys and chromosome 18 in mice and rats (Yaar, 1998).

1.3.4. The structural homologs of c-*fms*

C-*fms* has been classified as a member of the class III sub-class of the tyrosine kinase growth factor receptor family based on its homology to the PDGFr, and it is also related to epidermal growth factor (EGF), fibroblast growth factor (FGF), insulin, insulin-like growth factor (IGF-1) and c-*kit* receptors (Roussel, 1994). Table 1.3.4. represents different properties of c-*fms* compared to the PDGFr.

PROPERTIES	PDGFr β		c-*fms*	
Expression	Fibroblasts, Smooth Muscle Cells, Capillary Endothelial Cells, Neurones Meningeal Cells and Ito cells		Monocytes, Macrophages and their progenitors, B Cells, Osteoclasts, Placental Trophoblasts and in Smooth Muscle Cells from Rabbit Atherosclerotic lesions.	
Chromosomal Location	5q33-q35(human) C'some 18 in mouse		5q33.3-q34 (human) C'some 18 in mouse	
Amino Acid	Human	Mouse	Human	Mouse
• Precursor	1106	1098	972	976
• Mature	1074	1067	953	957
M_r (K)				
• Predicted	120.6	119.6	106	109
• Expressed	180	180	150	165
Potential N-linked glycosylation sites	11	11	11	9
Affinity K_d (M)	$\approx 10^{-10}$		$\approx 10^{-10}$	
Autophosphorylation Sites	Tyr579, 581, 740, 751, 771,857, 1009 and 1021 (human)		Tyr561, 571, 699, 708, 723 809 and 969 (human) Tyr559, 569, 697, 706, 721 807 and 967(murine)	

Table 1.3.4. Comparison of Properties associated with the PDGFr and c-*fms*.

The overall sequence homology that exists between the M-CSF and PDGF receptors, including spacing of Cys residues and short segments of sequence homology in the extracellular ligand-binding domain reflect a common ancestry and it is likely that one or the other arose from gene duplication (Roussel, 1994). A CLUSTALW 1.7 protein alignment of the PDGFr and c-*fms* illustrates the shared homology between these two growth factor receptors (Appendix A).

The cDNAs for murine, feline and human c-*fms* have all been cloned (Accession No. X06368, J03149 and X03663, respectively) (Roussel, 1994). The primary translation product of the human c-*fms* gene is a 972aa precursor peptide (976aa in murine cells) (Stanley *et al.*, 1997). The human c-*fms* precursor protein is composed of a 19aa signal peptide sequence which is post-translationally cleaved, a 493aa sequence corresponding to the extracellular domain, a 26aa transmembrane domain and a 434aa sequence coding for the cytoplasmic domain (Stanley *et al.*, 1997). After post-translational modification the mature form of the receptor is 953aa (957aa in murine cells) in length (Table. 1.3.4.) (Stanley *et al.*, 1997). The human c-*fms* is synthesised as an immature 130kDa transmembrane glycoprotein that undergoes modification via N-linked glycosylation at up to 11 canonical sequences (Asn-X-Ser/Thr) clustered in the amino-terminal extracellular domain (Sherr, 1990). The mature 150kDa cell surface form is generally produced within an hour following translation (Rettenmier *et al.*, 1988; Sherr, 1990). The mature murine receptor is larger (165kDa), possibly due to differences in the processing of the carbohydrate side-chains (Li and Stanley, 1991).

1.3.5. M-CSF activation of c-*fms*

In the absence of extracellular ligand c-*fms*, present at the cell surface, is turned over with a half-life of 2-3 hours after which down-regulation of receptor expression occurs (Sherr, 1988). This is then followed by a period of several hours of *de novo* synthesis during which receptors re-accumulate at the cell surface (Sherr, 1988). M-CSF stimulation of c-*fms* results in receptor internalisation and short-term replacement of plasma membrane receptor by mobilisation of intracellular pools of immature or newly synthesised c-*fms*, however the net effect of M-CSF stimulation is down-regulation of receptor expression (Munn and Cheung, 1992). When M-CSF

binds to c-*fms*, a non-covalent receptor dimerisation event proceeds in conjunction with an initial wave of receptor tyrosine phosphorylation (Li and Stanley, 1991; Tapley *et al.*, 1990). Receptor tyrosine dephosphorylation occurs concurrently with tyrosine phosphorylation of specific cytosolic proteins prior to extracellular, inter-chain disulphide linkage of receptor monomer subunits (Li and Stanley, 1991; Stanley *et al.*, 1994). Blockage of extracellular disulphide bonding inhibits receptor internalisation and causes an eight-fold increase in the rate of appearance of tyrosine phosphorylated proteins (Li and Stanley, 1991). A second wave of receptor tyrosine phosphorylation then occurs in conjunction with increased receptor serine phosphorylation and in the case of murine c-*fms* formation of either a 380kDa or 450kDa disulphide linked heterodimer occurs (Stanley *et al.*, 1994). These heterodimers are composed of the 165kDa receptor monomer and/or modified forms of the 165kDa monomer (215kDa and 250kDa) (Stanley *et al.*, 1994). Kinase inactivation then follows as a result of receptor dephosphorylation and poly-ubiquitination occurs prior to internalisation of the ligand-receptor complex (Stanley *et al.*, 1994). Ubiquitination is a prerequisite for degradation of the c-*fms* cytoplasmic domain (Stanley *et al.*, 1994). Internalisation of the ligand-receptor complex proceeds via clathrin coated vesicles, which are transported to secondary lysosomes for degradation of the receptor-ligand complex (Li and Stanley, 1991; Sherr and Stanley, 1990). Studies of a chimeric c-*fms*, in which the cytoplasmic tail of c-*fms* is fused to Glycophorin A suggest that structural features in the extracellular domain determine lysosomal targeting and degradation of internalised receptor (Lee and Nienhuis, 1992) Internalisation and degradation of the c-*fms* down-regulates the M-CSF signal (Ohtsuka *et al.*, 1990; Rettenmier *et al.*, 1988).

Therefore, ligand-induced non-covalent dimerisation activates the intrinsic protein kinase activity of c-*fms* and leads to covalent dimerisation of non-covalently associated receptor monomers. Dimeric c-*fms* is subsequently modified, resulting in inactivation of receptor kinase activity and receptor dephosphorylation prior to internalisation, ubiquitination, and trafficking to the lysosome (Li and Stanley, 1991; Stanley *et al.*, 1994).

1.3.6. The functional domains of c-*fms*

The extracellular domain of c-*fms* is composed of five immunoglobulin-like (Ig-like) loops designated $D_1 \rightarrow D_5$ from the amino-terminus (Fig. 1.3.6.) (Roussel, 1994). All the Ig-like domains of the receptor, with the exception of D_4 contain intra-domain disulphide bonds (Roussel, 1994). The M-CSF binding site consists of Loops $D_1 \rightarrow D_3$. Due to the absence of a disulphide bond in the D_4 loop the receptor possesses a degree of flexibility which permits conformational changes upon ligand binding which facilitate receptor dimerisation (Roussel, 1994).

The cytoplasmic tail of c-*fms* can be divided into five distinct sub-domains; the juxtamembrane region, the ATP-binding lobe, the kinase insert region, the main tyrosine kinase domain and the carboxyl-terminal tail (Fig. 1.3.6.) (Roussel, 1994). Upon activation, human c-*fms* is phosphorylated, on specific tyrosine residues, Tyr^{543}, Tyr^{561} and Tyr^{571} in the juxta-membrane region, Tyr^{699}, Tyr^{708} and Tyr^{723} within the kinase insert domain, Tyr^{809} within the core kinase domain and Tyr^{969} in the carboxyl tail region. (residues 541, 559, 569, 697, 706, 721, 807, 967 in murine cells, respectively) (Clark *et al.*, 1992; Courtneidge *et al.*, 1993; Joos *et al.*, 1996; Roussel *et al.*, 1990; Shurtleff *et al.*, 1990).

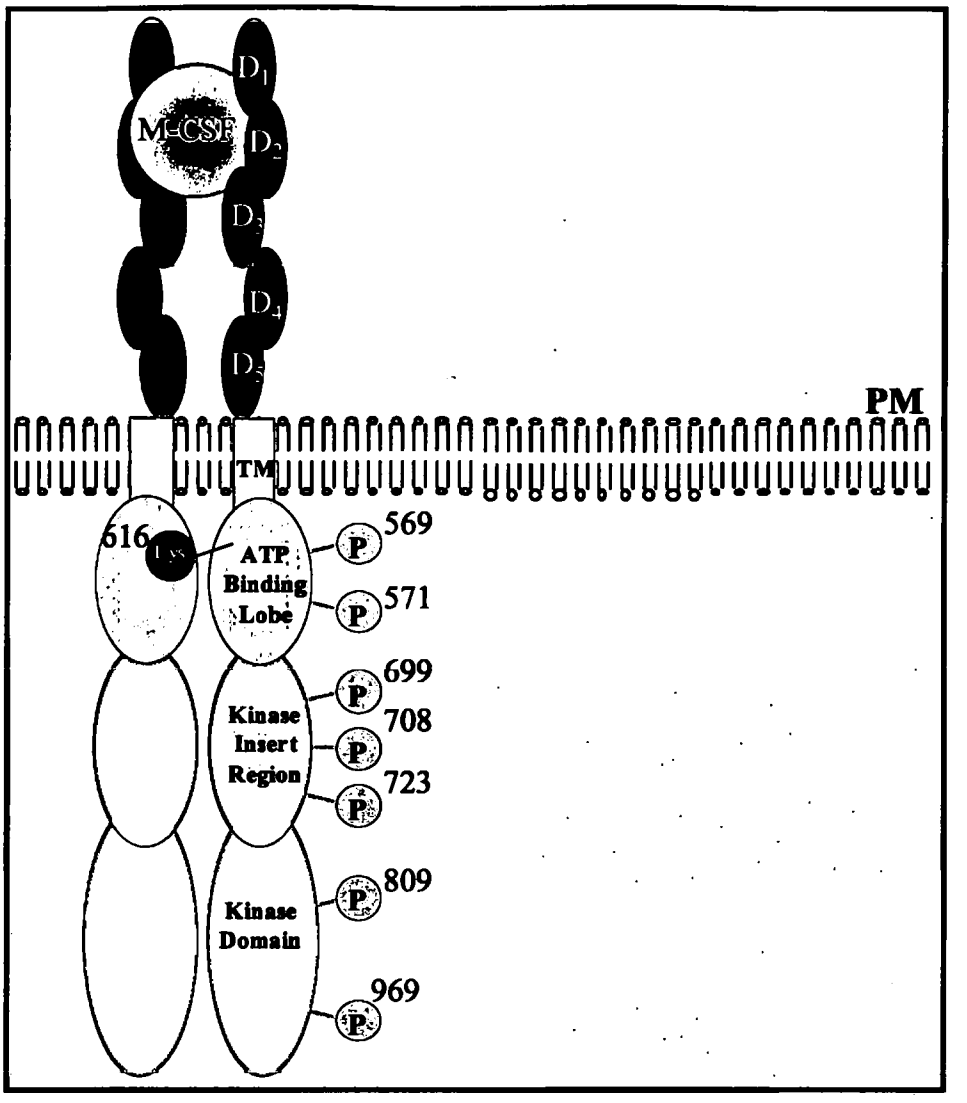


Fig. 1.3.6. Domain structure of the M-CSF receptor and important sites of tyrosine phosphorylation.

It has been demonstrated that in common with other RTK's, c-*fms* binds to *src*-family kinases (*src, fyn* and *yes*), Shc, Grb2 and PI 3-kinase (Alonso *et al.*, 1995; Cantley *et al.*, 1991; Courtneidge *et al.*, 1993; Rohrschneider *et al.*, 1997). Following c-*fms* activation in macrophages, various cellular proteins are transiently tyrosine phosphorylated, including Shc, Sos1, PTP-1C (SHP-1) and SHIP (Li and Stanley, 1991; Neel and Tonks, 1997; Yeung *et al.*, 1992). Shc, Sos1, SHIP and Grb2 have been shown to form complexes following receptor activation in myeloid cells and appear to be downstream effectors of numerous signalling mechanisms in addition to the c-*fms* signalling pathway (Rohrschneider *et al.*, 1997). Thus, although c-*fms* does

not bind directly with Shc, Sos1 and the 150kDa protein it does regulate their intracellular functions possibly through the Grb2 adapter protein (Stanley *et al.*, 1997). SHP-1, a protein tyrosine phosphatase, is activated downstream of various cytokine receptors and regulates the tyrosine phosphorylation state of cellular proteins (Neel and Tonks, 1997).

A more detailed review of c-*fms* signalling will be discussed in section 1.6.0., following a discussion of Phosphatidylinositol 3-Kinase (PI 3-kinase), a key component in signalling including that activated by M-CSF.

1.4.0. Phosphatidylinositol 3-kinase

1.4.1. Lipid signalling

When a stimulatory molecule binds to a receptor at the exterior of the cell membrane, second messengers such as those derived from membrane-associated phospholipids transduce signals to the interior of the cell (Toker and Cantley, 1997). Multiple phospholipid species are generated by the actions of various enzymes with intrinsic lipase, lipid kinase and lipid phosphatase activities such as phospholipase C and Phosphatidylinositol (PI) 3-kinase (Toker and Cantley, 1997). Kaplan *et al* were first to demonstrate that lipid second messenger signalling was associated with growth factor receptor activation and observed that a PI kinase activity was recruited into anti-phosphotyrosine immunoprecipitates following PDGF stimulation of NIH 3T3 cells or cells transformed with v-*fms* (Kaplan *et al.*, 1987).

1.4.2. Phosphoinositide Synthesis

A key event in growth factor signalling cascades is the metabolism of phosphoinositides (PI) (Kapeller and Cantley, 1994). Phosphoinositide kinases (PI-kinases) phosphorylate the D_3, D_4 and D_5 positions of the inositol ring of phosphatidylinositol (PI), (Fig. 1.4.2.), generating various polyphosphorylated PI species (Fig. 1.4.3.) (Kapeller and Cantley, 1994).

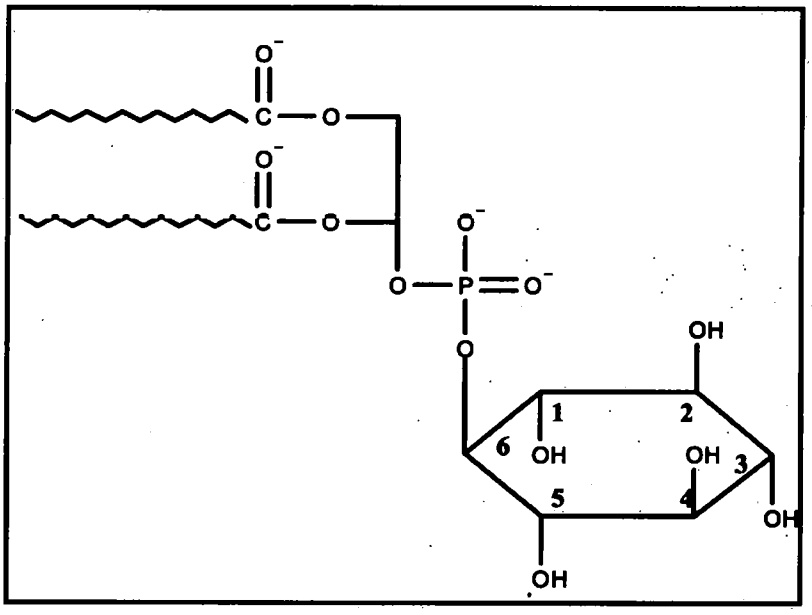


Fig. 1.4.2. The Structure of Phosphatidylinositol.

Growth factors tightly modulate the kinases, phosphatases and lipases involved in the regulation of phosphoinositide levels in cellular sub-compartments and two pathways are implicated in production of these phosphorylated lipids; the canonical phosphoinositide turnover pathway and the 3-phosphoinositide pathway (Kapeller and Cantley, 1994). In the canonical pathway, PI is sequentially phosphorylated by PI 4-kinase and PI_4P 5-kinase to generate $PI_{4,5}P_2$, the major substrate for phosphoinositide-specific phospholipase-C (PI-PLC) (Kapeller and Cantley, 1994) and PI 3-kinase (Fig. 1.4.3.).

The rapid activation of this PI-specific PLC activity in response to ligand-receptor interaction results in hydrolysis of $PI_{4,5}P_2$ generating two second messengers, inositol trisphosphate (IP_3) and diacylglycerol (DAG) (Fig. 1.4.3.) (Kapeller and Cantley, 1994). IP_3 promotes release of Ca^{2+} from intracellular stores and DAG activates protein kinase C (PKC) (Kapeller and Cantley, 1994). The increases in both intracellular [Ca^{2+}] and PKC activity are important in the step-wise propagation of signals from the cell surface to the nucleus (Kapeller and Cantley, 1994).

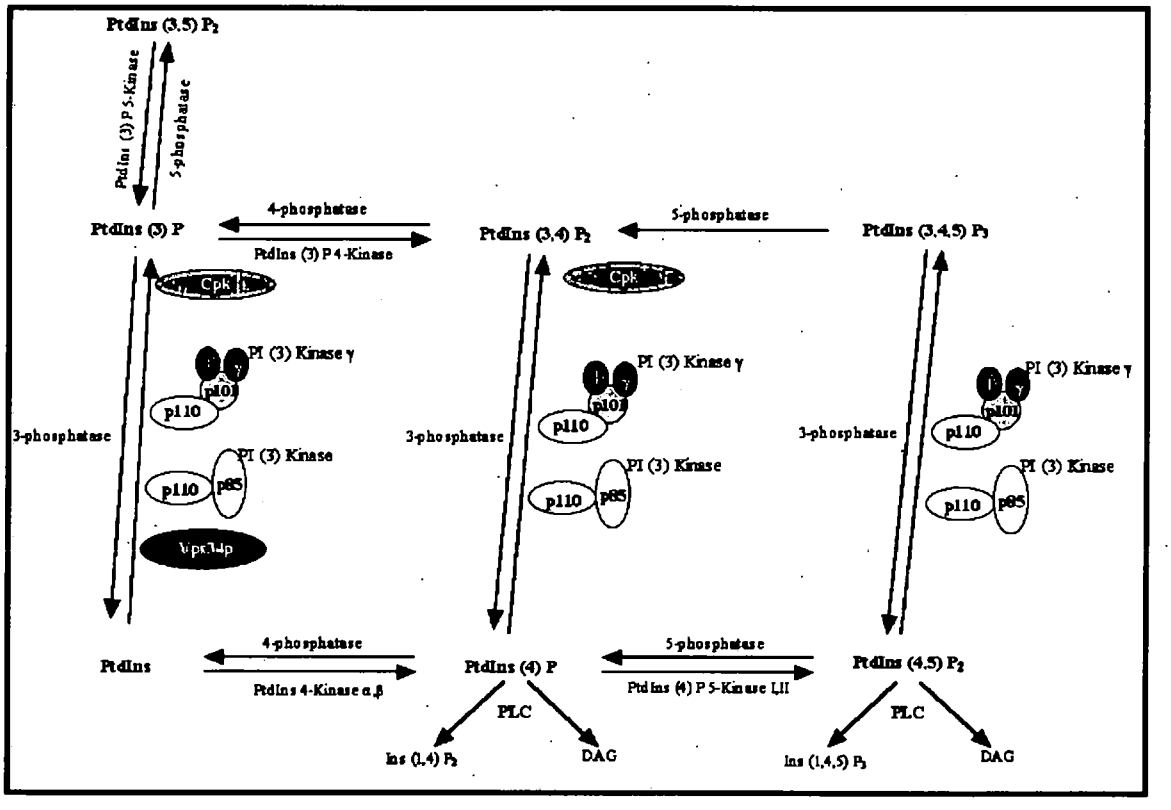


Fig. 1.4.3. Phosphoinositides and the enzymes involved in their metabolism.

For a number of years the canonical pathway was considered the only pathway employed in response to extracellular stimuli. PI 3-kinase was discovered over ten years ago and is described as an enzymatic activity capable of phosphorylating phosphatidylinositol (PI) at the D-3 position of the inositol ring to form PI_3P (Whitman *et al.*, 1988). This activity was originally found in an immunoprecipitated complex containing v-*src* and middle-T-antigen of the polyoma virus obtained from

transformed cells (Whitman *et al.*, 1988). Almost at the same time another phospholipid species, $PI_{3,4,5}P_3$ was isolated from neutrophils (Traynor-Kaplan *et al.*, 1988) and has since been demonstrated to be an important PI 3-kinase product, involved in various cellular responses.

1.4.3. PI 3-kinase

The PI kinase family has been divided into three classes; class 1, which contains the classical PI 3-kinases that produce $PI_{3,4,5}P_3$, class 2 PI 3-kinases, which cannot utilise $PI_{4,5}P_2$ as a substrate (Shibasaki *et al.*, 1993) and class 3 which have been identified as containing the PI-specific 3-kinases that generate PI_3P (Volinia *et al.*, 1995). Class 1 PI 3-kinases can be further subdivided into class 1a which contain the classical p85/p110 PI 3-kinases and class 1b which contain the G-protein-regulated PI 3-kinases (Shepherd *et al.*, 1998). PI 3-kinase activity is stimulated by various cytokines including IL-3, IL-4, IL-5, GM-CSF and SLF (Gold *et al.*, 1994). PI 3-kinase has an associated protein serine kinase activity which is inhibited by non-ionic detergents but not by adenosine, distinguishing it from other PI-kinases (Cantley *et al.*, 1991; Carpenter *et al.*, 1990).

PI, PI_4P and $PI_{4,5}P_2$ are all substrates of PI 3-kinase, and leads to the production of PI_3P, $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$, respectively (Fig. 1.4.3.) (Kapeller and Cantley, 1994). Products of the 3-PI pathway are implicated in various cellular mechanisms including mitogenesis, cell motility, vesicle trafficking, Ras activation, the secretory pathway and apoptosis which will be discussed in greater detail later (Cantley *et al.*, 1991; Valius and Kazlauskas, 1993).

Recently a novel phosphatidylinositol species has been isolated from murine fibroblasts, $PI_{3,5}P_2$ which is the product of an agonist-independent, wortmannin sensitive pathway in resting fibroblasts (Whiteford *et al.*, 1997). The formation and degradation of $PI_{3,5}P_2$ appears to be controlled by two enzymes, a PI_3P specific 5-kinase and a $PI_{3,5}P_2$ specific 5-phosphatase, respectively (Whiteford *et al.*, 1997). At present a mammalian function for $PI_{3,5}P_2$ has yet to be elucidated but in the yeast, *S. cerevisiae* and *S. pombe* and in Cos-7 cells $PI_{3,5}P_2$ is produced in response to changes in external osmotic pressure (Dove *et al.*, 1997).

PI 3-kinase lipid products can directly associate with proteins and it has been shown that $PI_{3,4,5}P_3$ can bind to the SH2 domains of *src* and p85 (Rameh *et al.*, 1995). The residues critical for SH2 domain binding to phosphotyrosine motifs do not appear to be required for lipid binding (Rameh *et al.*, 1995). PI 3-kinase products $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ also bind to the pleckstrin homology (PH) domain of PKB causing its translocation to the plasma membrane (Stephens *et al.*, 1998). This suggests that PI 3-kinase products are involved in recruitment or localisation of SH2 domain and PH domain containing proteins to areas of PI 3-kinase activity.

In growth factor receptor signalling the activation of PI 3-kinase is stimulated by the binding of ligand to the extracellular domain of RTKs such as the EGF, PDGF and c-*fms* (Hu *et al.*, 1992; Kaplan *et al.*, 1987). Receptor auto-phosphorylation at specific tyrosine residues in the cytoplasmic domain enables PI 3-kinase and other cytoplasmic effector proteins to bind and propagate the receptor signal (Chou *et al.*, 1987; Escobedo and Williams, 1988; McClain *et al.*, 1987; Moolenaar *et al.*, 1988). PI 3-kinase also associates with various PTKs and in some instances acts downstream of receptors that are not RTKs (Panayotou *et al.*, 1992).

1.4.4. PI 3-kinase activity in disease

Elevated PI 3-kinase activity has been detected in over 90% of colorectal carcinoma tumours compared to normal mucosa from the same patient (Phillips *et al.*, 1998). Effective inhibitors of PI 3-kinase inhibitors may be of therapeutic benefit in treatment of certain aggressive cancers. The most frequently observed mutation of the EGFr in human tumours is a deletion of exons 2-7, which yield a truncated receptor termed EGFr vIII (Moscatello *et al.*, 1998). EGFr vIII can transform NIH 3T3 fibroblasts and the relative frequency of detection in tumours suggests that it has a selective advantage upon tumour cells *in vivo* (Moscatello *et al.*, 1998). Although EGFr vIII is a constitutively active tyrosine kinase there are no observable increase in GTP-Ras levels or MAPK activity when expressed in NIH 3T3 cells (Moscatello *et al.*, 1998). However, there is an eight fold increase in detected PI 3-kinase activity in EGFr vIII transformed fibroblasts compared to only a three fold increase in cells expressing normal receptor (Moscatello *et al.*, 1998). Therefore activation of PI 3-kinase is a key mediator of EGFr vIII transformation of human tumours.

1.5.0. Structure and regulation of PI 3-kinase

1.5.1. The p85 adaptor/regulatory subunit of PI 3-kinase

Initial molecular characterisation of PI 3-kinase has revealed it is a heterodimer, consisting of an 85kDa adapter subunit (p85) (Ruiz-Larrea *et al.*, 1993) and a 110kDa catalytic subunit (p110) (Woscholski *et al.*, 1994). To date, four distinct mammalian p85 subunits, p85α, p85β, p85γ, and the homolog of vps15p have been discovered for which the cDNA's have been cloned (Daduang *et al.*, 1995; Escobedo *et al.*, 1991; Hu *et al.*, 1995; Otsu *et al.*, 1991; Panayotou *et al.*, 1997; Pons *et al.*, 1995; Skolnik *et al.*,

1991). A 60kDa drosophila homolog of the p85 subunit of PI 3-kinase has also been purified from a YxxM motif affinity matrix and co-precipitates Dp110, the drosophila homolog of p110 (Weinkove *et al.*, 1997).

Fig. 1.5.1a. represents a nearest neighbour tree obtained from a CLUSTALW 1.7 (Corpet, 1988) multiple alignment of the protein sequences available for the homologs of p85 from mammals, drosophila and yeast.

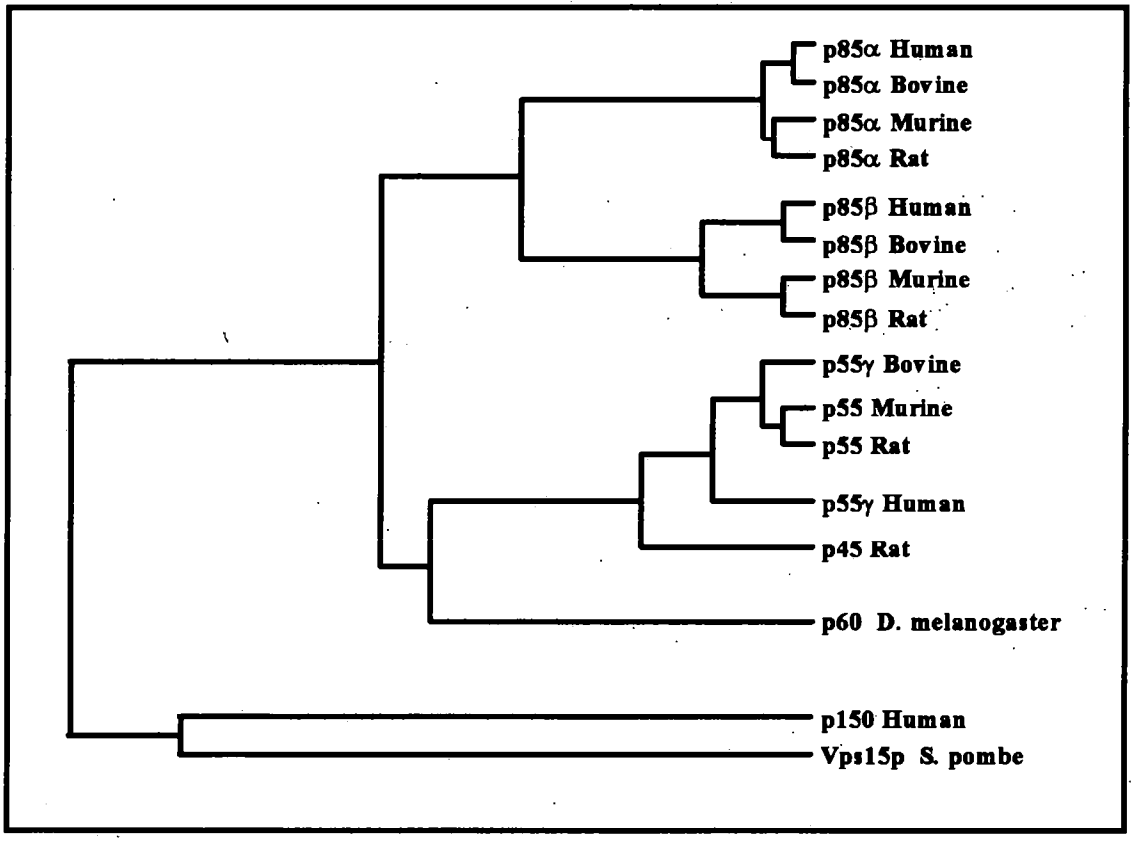


Fig. 1.5.1a. Nearest neighbour tree representing the homologies shared between PI 3-kinase regulatory subunits.

Both p85α and p85β are 724aa polypeptides with two SH2 domains and an amino-terminal *src*-homology 3 (SH3) domain (Fig 1.5.1b.) (Daduang *et al.*, 1995; Piccione *et al.*, 1993). Using a yeast two hybrid system the minimal determinant of p110 subunit association with p85 was mapped to a 192 amino acid portion between the N and C-terminal SH2 domains of p85, residues 429-621 and has the predicted structure

of an independently coiled-coil of two long anti-parallel α helices (Dhand *et al.*, 1994; Holt *et al.*, 1994; Hu *et al.*, 1993; Klippel *et al.*, 1993; Zhang *et al.*, 1993).

The p85 inter-SH2 domain alone is not sufficient on its own to stimulate PI 3-kinase activity and requires either one or both SH2 domains as well as the inter-SH2 domain to reconstitute PI 3-kinase activity *in vitro* (Holt *et al.*, 1994). SH2 domains were originally identified as conserved regulatory regions in *src* family-like kinases (Sadowski *et al.*, 1986), and mediate the associations of various proteins including PLCγ, Ras-GAP and adapter proteins such as Grb2, Shc and p85 (Marengere and Pawson, 1994). SH2 domains were initially characterised as an amino acid sequence of approx. 100aa that possessed no catalytic activity (Marengere and Pawson, 1994; Sadowski *et al.*, 1986).

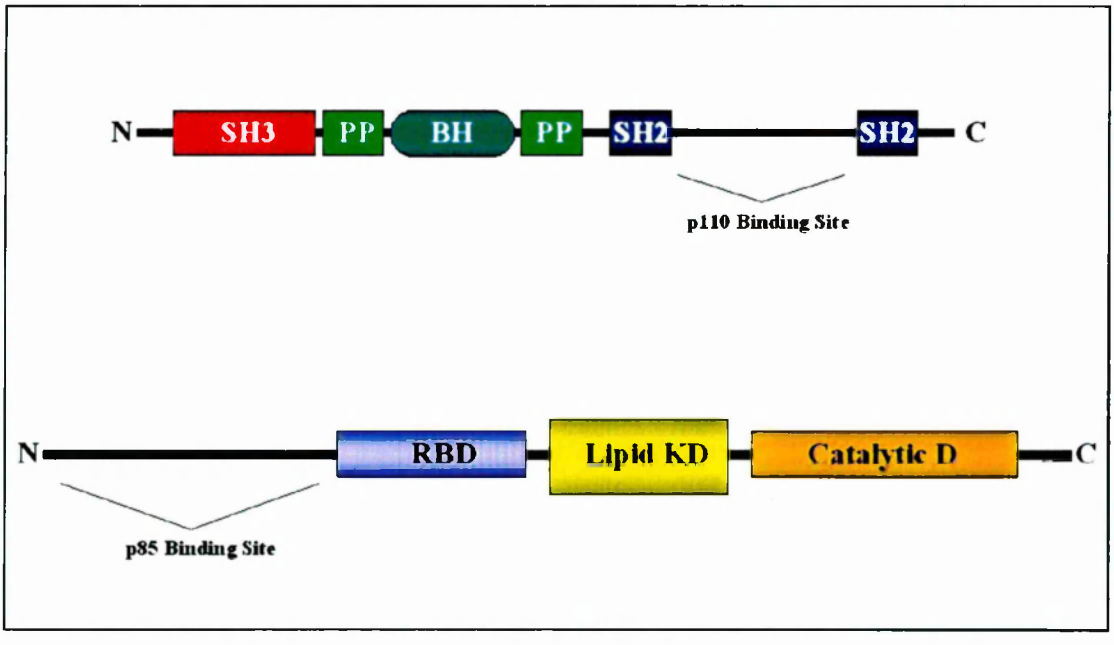


Fig. 1.5.1b. Diagram of Functional Domains Found in p85 and p110 Subunits of PI 3-kinase.

It has been demonstrated that synthetic phosphopeptides introduced into fibroblasts block the binding of PI 3-kinase to both PDGFr and polyoma middle T (Escobedo *et al.*, 1991; Fantl *et al.*, 1992; Kazlauskas *et al.*, 1992; Piccione *et al.*, 1993; Yoakim *et al.*, 1992). Through the use of a degenerate phosphopeptide library the consensus

recognition sequence for the p85 SH2 domains was mapped (Songyang *et al.*, 1993). The p85 SH2 domains select peptides with either Met, Val, Ile, or Glu at the +1 position relative to the phosphotyrosine and methionine at the +3 position (eg. pY-M/V/I/E-X-M) (Songyang *et al.*, 1993). The binding affinities of these synthetic phosphopeptides to the SH2 domains of p85 are one order of magnitude greater than unphosphorylated peptides or +3 Met mutant peptides with K_D's of 0.3-3nM and 1-10 μM, respectively (Felder *et al.*, 1993).

Another feature located between the SH3 domain and the amino-terminal SH2 domain is a region homologous to the carboxyl-terminal part of the Break-cluster region (Bcr) gene product (Kapeller *et al.*, 1994; Liu *et al.*, 1993). The Bcr homology (BH) domain is flanked on either side by two proline rich motifs (Fig 1.5.1b.) (Kapeller *et al.*, 1994; Liu *et al.*, 1993). A speculative role for the BH domain of p85 has been proposed in the stimulation of GTP hydrolysis on Rho, Rac and/or cdc42 (See below) (Diekmann *et al.*, 1991; Matsuo *et al.*, 1996; Tolias *et al.*, 1995). The SH2 domains bind to specific phosphotyrosine-containing motifs and the SH3 domains bind to short amino acid sequences that are rich in proline residues (Kapeller and Cantley, 1994). Through these binding sites p85 mediates the association of p110, and its intrinsic kinase activity, with target proteins (Dhand *et al.*, 1994; Klippel *et al.*, 1993; Rickles *et al.*, 1994). The ubiquitously expressed drosophila p60 adapter subunit, possesses two SH2 domains and an inter-SH2 domain but lacks the SH3 domain and BCR homology (BH) domain which are found in p85 (Weinkove *et al.*, 1997).

The SH3 domain of p85 is a member of a family of non-catalytic protein modules approx. 60aa in length which are common to many proteins that also possess SH2

domains (Fig. 1.5.1b.) (Rickles *et al.*, 1994; Sadowski *et al.*, 1986). SH3 domains bind to two classes of recognition sequence; class I ligands contain the consensus sequence RXLPPδPXX, where δ is L for *src* and R for PI 3-kinase and class II ligands contain a XXXPPLPXR motif for both *src* and PI 3-kinase (Cohen *et al.*, 1995). All high affinity SH3 ligands identified to date have a conserved PXXP motif and the p85α SH3 domain of PI 3-kinase exhibits a preference for a RXXRPLPPLPPP which has been identified by screening a modified phage display library (Rickles *et al.*, 1994).

The SH3 domain of p85 is involved in mediating the association of PI 3-kinase with other proteins via association with proline rich motifs (Parker and Waterfield, 1992). SH3 domains are also present in a variety of cytoskeletal proteins such as spectrin, fodrin, myosin 1B and actin-binding protein (ABP-1) (Kapeller and Cantley, 1994). The SH3 domains of c-*abl* and Grb2 bind to 3BP1, a protein with regions of homology to sequences of Rho-GAP which also associates with PI 3-kinase (Hartley *et al.*, 1995; Wang *et al.*, 1995). The SH3 domains of c-*abl*, lck, lyn, fyn and *src* can also associate with the proline-rich sequences flanking the BH domain of p85 (Kapeller *et al.*, 1994; Liu *et al.*, 1993). This demonstrates that SH3 domain containing proteins have the ability to link PTKs to effector molecules that activate various downstream signalling pathways (Kapeller *et al.*, 1994; Liu *et al.*, 1993; Skorski *et al.*, 1995). The SH3 domain of p85 and its proline-rich target sequences play an important regulatory function by targeting PI 3-kinase activity to specific cellular compartments, linking PI 3-kinase activity to both upstream and downstream elements. It has also been observed that p85 can bind directly to the SH3 domains of Abl, Lck, Fyn, and p85 itself (Kapeller *et al.*, 1994). Kapeller *et al* have demonstrated that two proline-rich sequences in the N-terminal region of p85 mediate

its interaction with SH3 domains which suggests that the SH3 domain of p85 may associate with other p85 subunits, or possibly fold back upon itself forming a self-association with the proline-rich motifs of the same subunit. This property of the p85 subunit may be part of the PI 3-kinase regulatory mechanism and recent data has suggested that the specific conformation of p85 is important for regulation of PI 3-kinase activity (Yu *et al.*, 1998), while Pleiman *et al* have demonstrated that activation of PI 3-kinase occurs *in vitro* following the association of the SH3 domains of either lyn or fyn, two *src*-family kinases (Pleiman *et al.*, 1994).

The p85 subunit may also be involved in regulation and translocation of proteins other than p110. For example, in insulin receptor signalling the p85α subunit forms separate complexes with p62 and an IRS-1-GAP complex (Pleiman *et al.*, 1994; Sung *et al.*, 1994). Analysis has shown that the amino and carboxyl terminal SH2 domains of p85α associate with the insulin receptor and IRS-1 respectively and may provide a mechanism for the translocation of PI 3-kinase and possibly other proteins to the plasma membrane, into close proximity with the insulin receptor (Baltensperger *et al.*, 1994; Pleiman *et al.*, 1994; Sung *et al.*, 1994). In IGF-1 signalling p85 associates with IRS-1 and the IGF-1 receptor via both SH2 domains and is possibly mediated by IFN-α occurring after the initial phosphorylation event on IRS-1 (Baltensperger *et al.*, 1994; Kooijman *et al.*, 1995; Sanchez-Margalet *et al.*, 1995; Uddin *et al.*, 1995).

1.5.2. The catalytic activity of PI 3-kinase

To date, five human PI 3-kinase activities have been identified; p110α, p110β, p110γ, p110δ and the human homolog of vps34 (Fry *et al.*, 1992; Hu and Schlessinger, 1994; Schu *et al.*, 1993; Stoyanov *et al.*, 1995; Vanhaesebroeck *et al.*, 1997; Volinia *et al.*, 1995). Many more PI 3-kinase homologs have been cloned from other species and

Fig. 1.5.2. is a nearest neighbour tree obtained from a CLUSTALW 1.7 multiple alignment of the available p110 protein sequences and illustrates the homology between PI kinase activities isolated from mammals, plants, drosophila, slime mould and yeast.

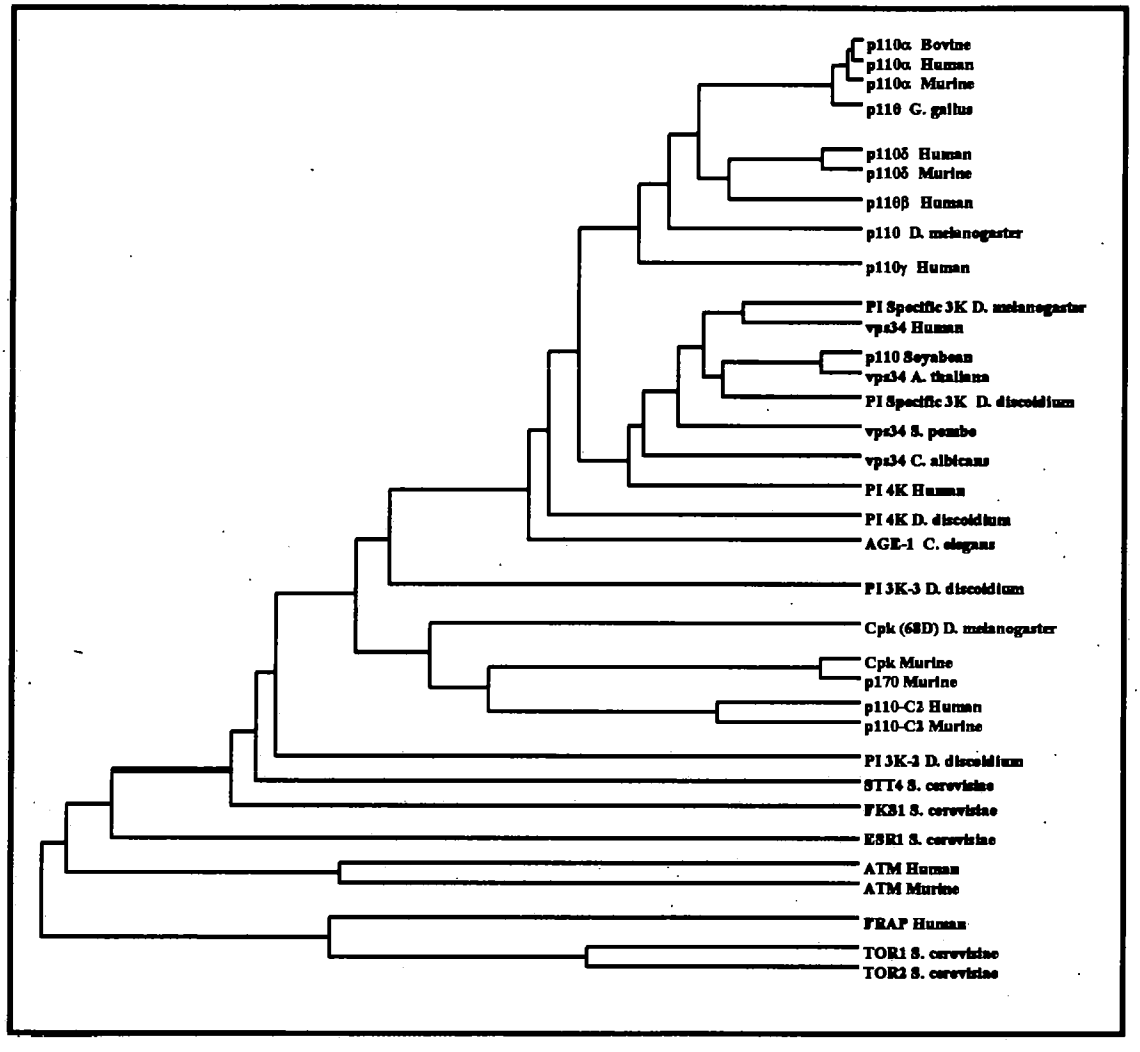


Fig. 1.5.2. Nearest neighbour tree representing the homologies shared between PI 3-kinase catalytic subunits.

1.5.3. The p110 catalytic subunit of PI 3-kinase

p110α and p110β, two related but distinct p85-associated 110kDa proteins have been identified in isolates of PI 3-kinase activity from rat liver (Fry *et al.*, 1992; Hiles *et al.*, 1992; Whitman *et al.*, 1988). The cDNA clones of both p110α and p110β were obtained from bovine brain libraries and they predict a mature protein of 1068aa. (Hiles *et al.*, 1992). Comparison of the predicted amino acid sequences of p110

isoforms with the catalytic domains of various protein kinases reveals several motifs (Fig. 1.5.1b). Residues at Asp^{916}, Asn^{921} and an Asp^{933}-Phe^{934}-Gly^{935} triplet may be homologous to residues present in the VI and VII catalytic subdomains of cyclic AMP-dependent protein kinase and therefore is implicated in phosphotransferase activity (Fry *et al.*, 1992).

A third p110 isoform is p110γ, which shares close homology to both p110α and p110β but does not associate with p85 (Stoyanov *et al.*, 1995). Its activity is regulated by G-proteins and, for example, is activated by the α and β/γ subunits of the G-protein, transducin in myeloid cells (Stephens *et al.*, 1994; Stoyanov *et al.*, 1995). p110γ, in contrast to p110α and p110β also possesses a PH domain at its amino-terminus. PH domains are known to associate with the phosphorylated lipid products of PI 3-kinase activity and are involved in signalling downstream of PI 3-kinase, however its function in p110γ remains unclear (Stoyanov *et al.*, 1995; Zhou *et al.*, 1995).

A new class I homolog, p110δ, has recently been cloned and characterised from leukocytes (Vanhaesebroeck *et al.*, 1997). Like the p110α and p110β isoforms, p110δ also displays a broad phosphatidylinositol specificity and interacts with SH2/SH3 domain containing p85 proteins and also GTP-Ras (Vanhaesebroeck *et al.*, 1997). Although p110δ associates with the p85 adapter proteins like other class I isoforms, it does not phosphorylate p85 but harbours an intrinsic autophosphorylation capacity and also contains unique protein motifs (Vanhaesebroeck *et al.*, 1997). These motifs include potential protein-protein interaction modules in the catalytic domain such as poly-proline regions and a basic region-leucine zipper (bZIP)-like domain (Vanhaesebroeck *et al.*, 1997). Expression of this p110 isoform is restricted

to cells of haematopoietic origin which confers haematopoietic cells with the potential to stimulate all three class I PI 3-kinase activities (Vanhaesebroeck *et al.*, 1997). No other functions for p110δ have been ascribed to date but it has been observed that it is differentially expressed in migratory but not non-migratory platelets (Vanhaesebroeck *et al.*, 1997). This apparent requirement in a motile phenotype may hint to an involvement of p110δ during cytoskeletal events (Vanhaesebroeck *et al.*, 1997).

Several *S. cerevisiae* proteins have been discovered that also share homology to PI 3-kinase (Kapeller and Cantley, 1994; Stack and Emr, 1994; Takegawa *et al.*, 1995). The yeast PI 3-kinase homolog vps34p is critical in targeting soluble hydrolases to the yeast vacuole and for correct vacuolar protein sorting and is also required in vacuole morphogenesis during budding (Hiles *et al.*, 1992; Stack and Emr, 1994; Takegawa *et al.*, 1995). Unlike mammalian p110, vps34p is specific only for PI, with other 3-kinase products, $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ absent in yeast (Schu *et al.*, 1993; Stack and Emr, 1994). Vps34p also exhibits an intrinsic protein kinase activity similar to p110α (Stack and Emr, 1994). A mammalian, PI-specific 3-kinase, homologous to vps34p has been cloned which also specifically phosphorylates only PI, and therefore by analogy to its yeast counterpart, may be required for correct vesicle and protein trafficking in mammals (Schu *et al.*, 1993; Volinia *et al.*, 1995).

The cDNA of a homolog closely related to yeast Vps34p has been cloned from *Arabidopsis thaliana*, *At*VPS34 (Welters *et al.*, 1994), and recently, PI3K_59F, a drosophila homolog of vps34/PI specific 3-kinase, has also been cloned (Linassier *et al.*, 1997). The other yeast PI 3-kinase homolog, TOR2, is a target protein for the rapa*myc*in-FKBP12 receptor complex (Kapeller and Cantley, 1994). Since rapa*myc*in causes G_1 arrest in both T cells and yeast, TOR2 and its non-essential homolog TOR1

may be required for maintaining progression through G_1 phase (Kapeller and Cantley, 1994). However no associated PI kinase activity has yet been found for TOR1 or 2.

In both drosophila and mouse another new class of PI 3-kinases has been discovered (Molz *et al.*, 1996; Virbasius *et al.*, 1996). In murine cells the proteins p170 and Cpk and in drosophila dCpk (68DV) share homology to p110 but differ from p110 at their N- and C- termini, possessing a C2 domain at their C- termini and lacking N- terminal p85 and Ras binding domains (Molz *et al.*, 1996; Shepherd *et al.*, 1998; Virbasius *et al.*, 1996). Closely related to these C2 domain containing proteins are human and murine p110-C2 which have only recently been cloned (Brown *et al.*, 1997; Misawa *et al.*, 1998). C2 domains are similar to the phosphoinositide-binding domains of PKC isoforms, and synaptotagmins (Molz *et al.*, 1996). C2 domain containing PI 3-kinases appear to phosphorylate the D3 position of the inositol ring of phosphoinositides but are selective for both PI and to a lesser extent PI 4-P but do not phosphorylate PI 4,5-P_2 which makes them unique PI 3-kinases (Molz *et al.*, 1996; Virbasius *et al.*, 1996). One other distant relative of PI 3-kinase is the Ataxia telangiectasia gene product, (ATM), is a DNA-dependent protein kinase (DNA-PK) which shows sequence homology with PI 3-kinase, but is more closely related to TOR1 and TOR2 of S. *cerevisiae* (Fig. 1.4.8.) (Lavin *et al.*, 1995; Savitsky *et al.*, 1995). Although Ataxia telangiectasia is a severely debilitating disease, clinically characterised by cerebellar degeneration, immunodefficiency, cell-cycle abnormalities and chromosomal instability, little is known about the function of ATM at present (Lavin *et al.*, 1995; Savitsky *et al.*, 1995).

1.5.4. PI 3-Kinase Inhibitors

PI 3-kinase inhibitors have been instrumental in the discovery of various properties of PI 3-kinase and receptor signalling. One such inhibitor, wortmannin, a fungal metabolite, has been used extensively to investigate the role of PI 3-kinase (Fig. 1.5.4.) (Woscholski *et al.*, 1994). It is known that wortmannin inhibits the PI 3-kinase of p110α with a low nanomolar IC_{50} but has no inhibitory effect on mammalian PI 4-kinase or S. *pombe* Vps34p (Vlahos *et al.*, 1994; Wymann *et al.*, 1996). Wortmannin was originally thought to specifically inhibit PI 3-kinase activity but this has recently been brought into doubt by evidence that wortmannin also inhibits Phospholipase A_2 (PLA_2) activity in fibroblasts (Cross *et al.*, 1995). Wortmannin binds to PI 3-kinase via formation of an enamine bond which couples the furan ring of wortmannin (labelled with red hatched box in Fig. 1.5.4.) with Lys^{802} in the lipid binding site on p110 (Wymann *et al.*, 1996). This lysine residue appears to be essential in the transfer of phosphate to the PI substrate of PI 3-kinase (Wymann *et al.*, 1996). Once bound, wortmannin irreversably blocks both the lipid and protein kinase functions of PI 3-kinase (Arcaro and Wymann, 1993; Barker *et al.*, 1995; Martys *et al.*, 1996).

Another PI 3-kinase inhibitor, LY294002 (Fig. 1.5.4.), (2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one) is not as potent as wortmannin, however it competes for the ATP-binding site on the p110 catalytic subunit which makes its mechanism of action distinct to that for wortmannin (Reif *et al.*, 1996; Vlahos *et al.*, 1994; Woscholski *et al.*, 1994). In addition, wortmannin is relatively unstable, whereas LY294002 is far more chemically stable and can be used for assays which require long incubations (Vlahos *et al.*, 1994). Wortmannin and LY294002 inhibit both lipid and protein kinase activities of PI 3-kinase at concentrations similar to that obtained for the p110α isoform *in vitro* (Vanhaesebroeck *et al.*, 1997). The IC_{50} for wortmannin is in the low

nanomolar range (1-10nM) (Arcaro and Wymann, 1993), whereas the IC_{50} for LY294002 is found in the low micromolar range (0.1-10μM) (Vlahos *et al.*, 1994).

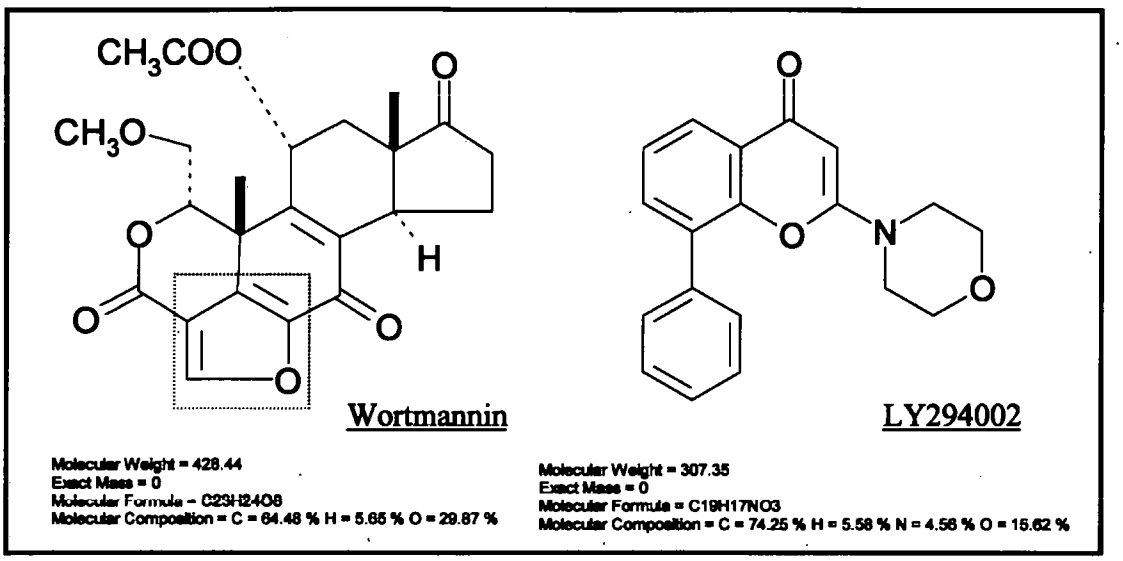


Fig. 1.5.4. Chemical structure of PI 3-kinase inhibitors wortmannin and LY294002.

Wortmannin is a potent and specific inhibitor for PI 3-kinase activity when used at concentrations comparable to its IC_{50} value. However at higher concentrations its specificity has been drawn into question and therefore interpretation of data obtained from inhibitor studies must be done carefully and whilst always bearing in mind the non-specific effects of PI 3-kinase inhibitors. It has been demonstrated that wortmannin blocks various signalling pathways *in vivo* and *in vitro*, including vesicle trafficking, cell adhesion, cytoskeletal and actin rearrangements and differentiation in a variety of cell types including, adiopocytes, erythroid cells and macrophages (Kilgour *et al.*, 1996; Tomiyama *et al.*, 1995; Vemuri *et al.*, 1996; Zell *et al.*, 1996).

1.5.5. Regulation of PI 3-Kinase Activity

As previously mentioned, the activity of PI 3-kinase has been found to increase in response to growth factors including M-CSF and PDGF (Cantley *et al.*, 1991; Herbst *et al.*, 1995). PI 3-kinase activity is also regulated by NRTKs (e.g. *fyn, yes* and *src*) in response to cellular activators such as Lipopolysaccharide (LPS) (Cantley *et al.*, 1991; Herrera-Velit and Reiner, 1996). The association of p85 with activated receptors, promotes the localisation of PI 3-kinase to the plasma membrane, bringing the p110 subunit into close proximity with its substrates (Rordorf-Nikolic *et al.*, 1995). The association of both SH2 domains of p85 with activated receptors appears essential for PI 3-kinase activation since doubly phosphorylated peptides, which mimic the receptor binding domain for p85, are more effective *in vitro* at activating PI 3-kinase than singly phosphorylated peptides (Kapeller and Cantley, 1994). Full occupancy of both amino and carboxyl terminal SH2 domains of p85 are required to produce full activation of PI 3-kinase activity by growth factors, since removal of either or both SH2 domains results in either a 50% reduction or complete abrogation of kinase activity (Rordorf-Nikolic *et al.*, 1995).

This may be due to conformational changes in the p85 subunit which in turn influences/activates the catalytic activity of p110, and recently it has been demonstrated that the conformation and physical properties of p85 may regulate p110 activity *in vitro* (Yu *et al.*, 1998). In sf9 insect cells infected with a baculovirus containing the p110 subunit, p110 is expressed as an active monomer whose activity is inhibited by co-expression of the p85 subunit (Yu *et al.*, 1998). Insect cells are routinely cultured at 28-30°C and addition of a bulky N-terminal Glutathione S-transferase (GST) tag to p110 or expression in HEK 293 cells at 30°C also abrogated the requirement for p85 (Yu *et al.*, 1998). These data suggest that the p85 regulatory

subunit may function to stabilise the overall conformation of p110, rather than by inducing a specific activated conformation. A physically bulky structure at the N-terminus of p110, whether it be p85 or a GST tag, appears to stabilise and activate, in part, p110 catalytic activity. Incubation at temperatures at or below 30°C may also permit stabilisation of the p110 monomer and therefore may have important implications for *in vitro* lipid kinase assays.

PI 3-kinase activity is also negatively regulated by an intrinsic serine/threonine kinase activity present on the p110 catalytic subunit, which phosphorylates p85 on serine and results in an 80% inhibition of activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994; Kapeller and Cantley, 1994). N-terminal sequence analysis has shown that phosphorylation of Ser^{608} in p85 is critical for regulation of PI 3-kinase activity (Carpenter *et al.*, 1993; Dhand *et al.*, 1994). Inhibition of PI 3-kinase activity by its intrinsic protein kinase can be reversed by treatment, *in vitro*, with Protein Phosphatase 2A (PP2A), which may be involved, to some degree, in the basal level activation of PI 3-kinase present in the cytosol but this remains to be conclusively determined *in vivo* (Carpenter *et al.*, 1993; Dhand *et al.*, 1994). In T-cells, p85α, when bound to p110, also appears to be differentially phosphorylated on serine and threonine residues compared to p85β which suggests a divergent function and regulatory role for p85α and p85β in T cells (Reif *et al.*, 1993). Therefore PI 3-kinase regulation may not only depend on the specific isoforms of p85 and p110 present and the subcellular compartment to which they are recruited during activation but also appears to depend on cell type and species differences. Therefore p85 subunit isoforms confer specificity during the association of specific PI 3-kinase activity with various specific substrates and adapter molecules, *in vivo* (Dhand *et al.*, 1994; Parker and Waterfield, 1992; Pleiman *et al.*, 1994; Reif *et al.*, 1993).

1.6.0. Cellular Functions of PI 3-Kinase

Studies using employing PI 3-kinase inhibitors, dominant-negative and constitutively active PI 3-kinase mutants have indicated four functional areas, (Fig. 1.6.1.), in which PI 3-kinase is involved;

1. Proliferation
2. Survival/Apoptosis
3. Intracellular vesicle trafficking/secretion
4. Regulation of the cytoskeleton

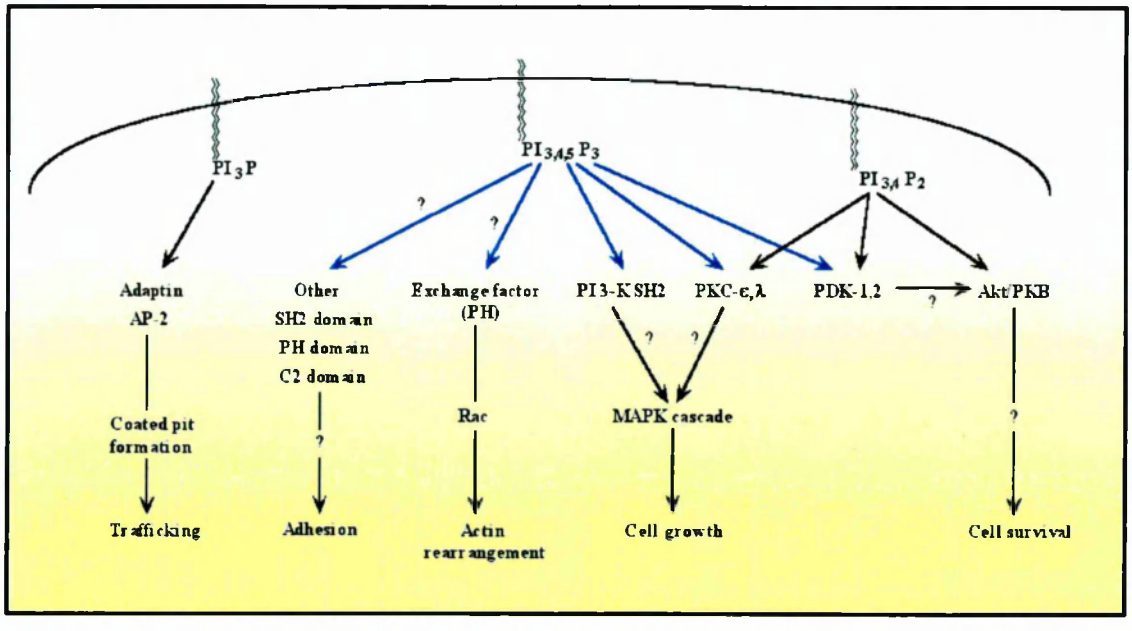


Fig. 1.6.1. Role of PI 3-kinase in cellular function.

1.6.1. Mitogenesis

PI 3-kinase is involved in mitogenic signalling in a cell type and stimulus dependent manner. The requirement for PI 3-kinase activity during growth factor receptor-mediated mitogenic signalling is not explicitly clear because of contradictions in the published data. It has been demonstrated that p85/p110α PI 3-kinase activity is required by the PDGF receptor for induction of DNA synthesis in NIH 3T3 cells (Roche *et al.*, 1994). However, in a separate study expression of a mutant PDGFr in either NIH 3T3 or 32D haematopoietic cells, which is unable to associate with PI 3-

kinase, did not impair PDGF stimulated mitogenesis (Yu *et al.*, 1994). In addition, PI 3-kinase is not required for the propagation of a mitogenic signal by G-protein coupled receptors Bombesin and Lysophosphatidic Acid (LPA) (Roche *et al.*, 1994) or for thrombin stimulated mitogenesis in vascular smooth muscle cells (Weiss and Apostolidis, 1995).

A polyclonal antibody (pAb), raised against the N-SH2 of p85α, has been shown to activate PI 3-kinase activity when microinjected into CHO cells and stimulates a three fold increase in PI 3-kinase activity of recombinant dimers, *in vitro* (McIlroy *et al.*, 1997). Activation of PI 3-kinase is sufficient to stimulate DNA synthesis in quiescent CHO cells and this activity requires both the p21 Ras/MAPK pathway and $p70^{S6K}$, a protein essential for progression through G_1 phase of the cell cycle (Alessi *et al.*, 1998; McIlroy *et al.*, 1997).

Activation of $p21^{ras}$ (Ras), a small molecular weight G-protein, is central to regulation of cell proliferation stimulated by various receptors, including the PDGFr and EGFr (Valius and Kazlauskas, 1993; Wiese *et al.*, 1995). The Ras protein family is a group of small monomeric GTP-binding proteins which are key regulators in cell proliferation, capable of causing cellular transformation when constitutively activated by the presence of point mutations (Rodriguez-Viciana *et al.*, 1996). In general, Ras has low intrinsic GTPase activity, which is stimulated by GTPase activating proteins (GAPs) including p120Ras•GAP (van der Geer *et al.*, 1997). PDGF stimulation of $GAP^{(-/-)}$ fibroblasts induces abnormal increases in GTP-Ras levels and the duration of MAPK activation compared to normal cells with tyrosine phosphorylation of p190Rho•GAP dependent on functional p120Ras•GAP (van der Geer *et al.*, 1997). Therefore p120Ras•GAP appears to downregulate activation of Ras and the MAPK

pathway following growth factor stimulation and also modulates phosphorylation of p190Rho•GAP but does not appear to be involved in mitogenic signalling in this model (van der Geer *et al.*, 1997).

Whether PI 3-kinase is an upstream or downstream effector of Ras is not completely clear. To date, it has been shown that GTP-bound Ras associates with both p110α and p110β subunits of PI 3-kinase involving Lys^{227} of p110 (Rodriguez-Viciana *et al.*, 1996). Association of Ras with PI 3-kinase may also be mediated indirectly through the Grb2•Sos complex via the Grb2 SH2 domain which can associate with PI 3-kinase (Kodaki *et al.*, 1994; Saleem *et al.*, 1995). Therefore Grb2 functions as an adapter molecule, binding Sos-associated Ras and then PI 3-kinase, thereby bringing Ras and PI 3-kinase within proximity of one another (Hu *et al.*, 1995; Saleem *et al.*, 1995). In COS-7 cells binding of tyrosine containing phosphopeptides to p85 occurs synergistically with GTP-Ras binding, suggesting that in Cos-7 cells activation of PI 3-kinase is dependent on Ras (Rodriguez-Viciana *et al.*, 1996). A decrease in Ras association with PI 3-kinase resulted in a reciprocal increase in basal levels of PI 3-kinase activity (Rodriguez-Viciana *et al.*, 1996). Introduction of a dominant-negative Ras mutant into NIH 3T3 cells also prevents the PDGF-dependent increase in cellular lipid levels suggesting, PI 3-kinase is a downstream effector of Ras (Carpenter and Cantley, 1996; Hu *et al.*, 1995).

The small GTPase R-ras displays a less potent transforming activity than the closely related Ras oncogene product but, like Ras, it can activate PI 3-kinase *in vitro*, but unlike Ras, R-ras cannot activate Raf-1 or MAPK in fibroblasts (Marte *et al.*, 1997). In co-transfection assays, Akt is effectively stimulated by either R-ras, Ras, mutants of Ras which can only activate PI 3-kinase or constitutively active PI 3-kinase (Marte

et al., 1997). Moreover, constitutive activation of PI 3-kinase, although sufficient for Akt activation, is not capable of activating MAPK, Jnk/SAPK or Erk (Marte *et al.*, 1997). Thus it appears that although Ras can activate both Raf-1/MAPK and PI 3-Kinase/Akt pathways, R-ras can only activate the PI 3-kinase/Akt pathway (Marte *et al.*, 1997).

Protein Kinase C (PKC) isoforms such as PKCε or PKCλ are activated directly by $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ and may lead to activation of Ras/Raf pathway (Toker and Cantley, 1997). PKCδ specifically associates with PI 3-kinase in platelets following cytokine stimulation, however although the PI 3-kinase inhibitor LY294002 did not inhibit this association, wortmannin did (Ettinger *et al.*, 1996). It is possible that while wortmannin specifically targets the lipid binding pocket (Wymann *et al.*, 1996), LY294002 inhibits PI 3-kinase protein kinase activity by binding within the ATP-binding site (Vlahos *et al.*, 1994). Therefore, it is likely that PKCδ association with PI 3-kinase possibly requires only substrate binding and not lipid kinase activation since the only difference between wortmannin and LY294002 are their respective target sites on PI 3-kinase.

1.6.2. Apoptosis

It is known that PI 3-kinase is an integral part of pathways by which insulin and NGF prevent apoptosis (Minshall *et al.*, 1996). PI 3-kinase products are involved in activation of PKB (Akt), a homolog of PKC and the cellular homolog of viral v-Akt, which possesses a PH domain that binds to PI 3-kinase products with high affinity (Alessi *et al.*, 1997). PKB activation in various cell lines has been shown to stimulate survival/proliferation, differentiation, GLUT4 translocation, Glycogen Synthase Kinase 3 (GSK3) downregulation and upregulation of E2F transcription factor

expression (Marte and Downward, 1997). Four PKB isoforms which bind to PIP_3 containing vesicles have been cloned to date; PKBα, PKBβ1 and PKBβ2 in humans and PKBγ in rats (Marte and Downward, 1997). Recently Alessi *et al* have isolated a PIP_3-dependent protein kinase-1 (PDK-1) from rabbit skeletal muscle which relies on the PI 3-kinase product, PIP_3 for activation (Alessi *et al.*, 1997). PDKs contain an N-terminal catalytic domain and a C-terminal PH domain which facilitates their association with PI 3-kinase products (Stephens *et al.*, 1998). PDK-1 phosphorylates PKB on Thr^{308} and Ser^{473} in response to insulin or IGF-1 stimulation (Alessi *et al.*, 1997). PDK-1 activation *in vitro* by $PI_{3,4,5}P_3$ and $PI_{3,4}P_2$ is not inhibited by wortmannin, and confirms that PDK-1 is not a PI 3-kinase family member (Alessi *et al.*, 1997). It also appears that phosphorylation and activation of $p70^{S6K}$ *in vivo* and vitro, a role previously ascribed to PKB, may also be performed by PDK-1 (Alessi *et al.*, 1998). Cells which require growth factors for their survival also require PI 3-kinase activity, but this appears to be mediated via PKB and not $p70^{S6K}$ (Yao and Cooper, 1996). It is interesting to note that PKB isoforms are over-expressed in various ovarian, pancreatic and breast carcinomas therefore may play a part in cell transformation and aberrant growth responses in these tumours (Alessi *et al.*, 1997).

In vitro studies have demonstrated that vesicles containing the phospholipid $PI_{3,4}P_2$ specifically bind to PKB via its PH domain and causes it to dimerise resulting in its activation (Franke *et al.*, 1997; Klippel *et al.*, 1997). Activation was not observed with vesicles containing PI_3P or $PI_{3,4,5}P_3$ and activation required functional PH domains on PKB (Klippel *et al.*, 1997). $PI_{3,4,5}P_3$ as well as $PI_{3,4}P_2$ cause translocation of PKB to the plasma membrane, thus enabling PDKs to phosphorylate and activate its kinase activity (Stephens *et al.*, 1998). Therefore the PI 3-kinase product $PI_{3,4}P_2$

and possibly $PI_{3,4,5}P_3$ provide a mechanism of recruitment for PKB to PDK-1 resulting in further PKB activation.

Both wortmannin and LY294002 induce apoptosis in healthy cells suggesting PI 3-kinase is also important in the survival pathway as well as in the differentiation pathway (Stoyanov *et al.*, 1995; Yao and Cooper, 1995). Serum starvation or incubation with PI 3-kinase inhibitors, wortmannin or LY294002, has been shown to induce apoptosis in PC12 cells, Rat-1 cells and REF52 cells but not in NIH 3T3 or Balb 3T3 cells (Yao and Cooper, 1996; Yao and Cooper, 1995). In addition, neuronal cells transfected with wild-type PDGFr enter into apoptosis in the absence of PDGF or when the receptor possesses a mutated PI 3-kinase binding site (Carpenter and Cantley, 1996). Ras activation of PI 3-kinase activity also leads to suppression of c-*myc*-induced apoptosis through PKB, but not $p70^{S6K}$ (Kauffmann-Zeh *et al.*, 1997). However, a complicating factor is that Ras is also an effective promoter of apoptosis through the Raf pathway (Kauffmann-Zeh *et al.*, 1997). In Rat-1 fibroblasts apoptosis induced by serum starvation cannot be rescued by Ras, Raf or *src* activation (Kennedy *et al.*, 1997). However protection against apoptosis is conferred by PKB activation, and apoptosis is accelerated by PI 3-kinase inhibitors wortmannin and LY294002 (Kennedy *et al.*, 1997).

A dominant negative, kinase-deficient PKB can inhibit BCR/ABL-dependent transformation *in vitro* and suppresses leukaemia development in SCID mice (Skorski *et al.*, 1997). Inhibition of PI 3-kinase activity with wortmannin also suppresses the BCR/ABL-dependent colony formation of murine bone marrow cells in culture suggesting that the PI 3-kinase/PKB pathway is essential for BCR/ABL leukaemogenesis (Skorski *et al.*, 1997). Yin *et al* have recently reported the

possibility that the p85 alone, without associated PI 3-kinase activity may participate in apoptosis induced by oxidative stress in a fibroblastic cell line (Yin *et al.*, 1998). However, this still remains to be confirmed.

1.6.3. Vesicle Trafficking

It was first demonstrated that PI 3-kinase activity was important for vesicle trafficking by the essential role for vps34p in late Golgi-vacuole trafficking in *S. cerevisiae* (Herman and Emr, 1990; Roth and Sternweis, 1997; Stack and Emr, 1994). Vps34p is known to associate with vps15p, which recruits vps34p to the plasma membrane where it is activated, vps15p appearing to function in an analogous manner to p85 (Stack and Emr, 1994). In mammalian cells there is substantial evidence for a separate but similar protein to vps15p which functions in trafficking of vesicles to lysosomes which are the mammalian functional equivalent of yeast vacuoles (Stack and Emr, 1994), and a 150kDa mammalian homolog of vps15 has recently been cloned (Panayotou *et al.*, 1997). Therefore there is a mechanism for sorting of vacuolar proteins which is highly conserved between yeast and mammals. Wortmannin has been demonstrated to block Golgi to lysosome trafficking of Pro-cathepsin D, resulting in the accumulation of large vesicle-like structures similar to pre-lysosomes (Davidson, 1995; Joly *et al.*, 1995). This implies PI 3-kinase may regulate trafficking of lysosomal enzymes, possibly by interference with the mannose 6-phosphate receptor-dependent sorting event in the trans-golgi network (Brown *et al.*, 1995). Since PI-specific 3-kinase is homologous to vps34p and so only produces one lipid product it has been proposed that it and its product, PI_3P, are important in the correct control of trans-golgi network-lysosome trafficking, late endosome - lysosome trafficking and endosomal recycling (Rameh *et al.*, 1995). However, although wortmannin inhibits PI 3-kinase activity causing an almost complete

decrease in cellular levels of $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$, there is only a 70% decrease in the levels of PI_3P suggesting that the PI-specific 3-kinase is wortmannin insensitive and continues to synthesise PI_3P (Zell *et al.*, 1996). Therefore $PI_{3,4}P_2$ and/or $PI_{3,4,5}P_3$ are also involved in late endosome to lysosome trafficking and endosomal recycling (Downward, 1995) and $PI_{3,4,5}P_3$ may be involved the activation of an ARNO/cytohesin-1/Grp-1 family member which regulates the assembly of coated vesicles (Toker and Cantley, 1997). In *D. discoidium* deficient for the two p110 homologs DdPIK1 or DdPIK2, there is impaired vesicle trafficking as well as a disorganised actin cytoskeleton (Roth and Sternweis, 1997).

The most compelling evidence in support of a role for PI 3-kinase activity during vesicle trafficking in mammalian cells comes from studies conducted with PDGFr mutants (Zell *et al.*, 1996). Mutation of the PI 3-kinase binding site on the PDGFr expressed in fibroblasts results in blockage of PDGF-dependent receptor lysosomal degradation (Shpetner *et al.*, 1996). In addition detailed study carried out in fibroblasts the has shown that in the presence of wortmannin, trafficking of the internalised PDGF receptor is arrested at a juxtamembrane site, prior to reaching the sorting endosome (Joly *et al.*, 1995). This suggests that trafficking of internalised PDGFr and its degradation is dependent on products of a PI 3-kinase activity rather than a PI-specific 3-kinase.

Inhibitors of PI 3-kinase also block endocytosis in macrophages by preventing completion of vesicle formation, although initiation of vesicle still occurs (Araki *et al.*, 1996). Wortmannin and LY294002 inhibit fluid-phase pinocytosis and Fc receptor mediated phagocytosis, but have little effect on receptor mediated endocytosis of LDL (Araki *et al.*, 1996). Wortmannin also partially inhibited

transferrin receptor internalisation and endosome recycling back to the cell surface in CHO cells (Martys *et al.*, 1996). Therefore it appears that PI 3-kinase activity is required at three stages; internalisation; transit from early endosomes to the recycling and lysosomal compartments; and transit from recycling compartments to the cell surface (Martys *et al.*, 1996).

1.6.4. The Cytoskeleton

$PI_{4,5}P_2$ is an important molecule in the regulation of the actin cytoskeleton (Toker and Cantley, 1997; Welch *et al.*, 1997). Studies in fibroblasts demonstrate that $PI_{4,5}P_2$ can promote actin filament growth by binding to gelsolin and other actin capping proteins thereby releasing these proteins from actin termini (Wymann and Arcaro, 1994), and synthesis of $PI_{4,5}P_2$ can be stimulated by addition of constitutively active Rac to permeabilised platelets (Parker, 1995). In addition, wortmannin does not inhibit thrombin dependent actin uncapping suggesting that PI 3-kinase activity may not be required for this effect. However PDGFr signalling in fibroblasts depends on PI 3-kinase for the activation of Rac (Parker, 1995). GTP binding to Rac is inhibited by wortmannin when cells are stimulated with PDGF, suggesting that Rac lies downstream of PI 3-kinase (Parker, 1995), and it has been reported that addition of constitutively active Rac reverses wortmannin inhibition in Swiss 3T3 cells (Bokoch *et al.*, 1996). The primary product of PI 3-kinase, $PI_{3,4,5}P_3$, which may remain associated with PI 3-kinase, causes GDP-Rac to dissociate from its binding protein Rac-guanine nucleotide dissociation inhibitor (Rac-GDI) again placing Rac downstream of PI 3-kinase (Bokoch *et al.*, 1996; Nobes *et al.*, 1995; Parker, 1995). Dissociation of Rac from Rac-GDI facilitates phosphate-transfer and leads to formation of membrane-associated GTP-Rac which stimulates actin polymerisation

and membrane ruffling or blebbing in PDGFr, IGFr, insulin and thrombin receptor signalling (Kundra *et al.*, 1994; Parker, 1995; Ridley *et al.*, 1992).

The PH-domain containing protein, profilin is involved in the regulation of actin assembly and interacts with $PI_{3,4,5}P_3$ and $PI_{3,4}P_2$ (Toker and Cantley, 1997). Binding of $PI_{3,4}P_2$ to profilin results in actin polymerisation, while $PI_{3,4}P_2$ also inhibits the actin severing activity of gelsolin and in permeabilised platelets has been shown to stimulate actin uncapping (Toker and Cantley, 1997). $PI_{4,5}P_2$ also binds to other proteins which regulate or bind to the actin cytoskeleton including, cap-2, and α-actinin as well as gelsolin, and possibly serves as an attachment site for PH-domain-containing proteins involved in actin regulation (Bae *et al.*, 1998; Rhee and Bae, 1997; Tapon and Hall, 1997). Therefore PI 3-kinase products may provide a more selective mechanism for inducing reorganisation of the actin cytoskeleton, whereas the ubiquitous $PI_{4,5}P_2$ may have more of a general house-keeping role in actin organisation. In addition to being a substrate for PI 3-kinase, $PI_{4,5}P_2$ is also a substrate for PLCγ, and hydrolysis of $PI_{4,5}P_2$ by PLCγ is one of the early events during signalling leading to the formation of DAG and IP_3 which activate PKC and calcium mobilisation, respectively (Bae *et al.*, 1998). Recently it has been demonstrated that the PI 3-kinase product $PI_{3,4,5}P_3$ activates PLCγ *in vitro* (Bae *et al.*, 1998). Therefore stimulation of PI 3-kinase activity results in activation of various pathways which all appear to have the net effect of depleting cellular $PI_{4,5}P_2$ levels which may also affect the organisation of the actin cytoskeleton.

Actin dynamics in lamellepodia are driven by continuous cycles of actin polymerisation, retrograde flow and actin depolymerisation (Welch *et al.*, 1997). The leading edge of a motile cell or cells responding to a chemotactic gradient are

composed of thin protrusions of membrane which continuously extend and retract, mediating the initial stage of cell movement whilst also determining the direction of eventual movement (Welch *et al.*, 1997). M-CSF stimulates actin reorganisation in BAC1.2F5 cells leading to formation of filopodia, lamellepodia and membrane ruffles as well as fine actin cables within the cell (Allen *et al.*, 1997). These changes in cell morphology are mediated by Rho family proteins that are regulated by a combination of GAPs, GEFs and GDIs (Allen *et al.*, 1997). Formation of lamellepodia, membrane ruffling and focal adhesion complexes are mediated by Rac which is also regulated indirectly by cdc42 which also regulates filopodia formation (Allen *et al.*, 1997). In turn Rac activation activates Rho leading to formation of actin cables (Allen *et al.*, 1997). Interestingly, the formation of filopodia negatively regulates further lamellepodia, focal complex and actin cable formation as well as membrane ruffling (Allen *et al.*, 1997). Expression of a constitutively active p110α PI 3-kinase in NIH 3T3 cells induces Rac-mediated lamellepodia and focal complex formation and Rho-mediated stress fibres and focal adhesion formation (Reif *et al.*, 1996). However PI 3-kinase activity is not required for Ras/Rac/Rho signalling pathways that lead to gene transcription thus PI 3-kinase activity acts selectively on Rho family GTPases to initiate some but not all of their responses (Reif *et al.*, 1996).

PI 3-kinase is believed to mediate the initial steps leading to increased membrane ruffling following activation of cell surface receptors such as those for PDGF and M-CSF (Araki *et al.*, 1996; Ruusala *et al.*, 1998). PI 3-kinase is also required for some forms of cell motility and adherence since fibroblasts containing PDGFr mutants which do not possess the PI 3-kinase binding site do not undergo plasma membrane ruffling, nor do they respond chemotactically to PDGF gradients (Chong *et al.*, 1994; Kundra *et al.*, 1994; Wennstrom *et al.*, 1994).

The Rho family proteins Rho and Rac are responsible for cell migration in response to M-CSF in the BAC1.2F5 macrophage cell line (Allen *et al.*, 1998). It is known that PI 3-kinase is an effector for Rho, since GTP-bound Rho stimulates the production of $PI_{3,4,5}P_3$ in platelet lysates (see below) (Stephens *et al.*, 1993; Zhang *et al.*, 1993). The other Rho family member, cdc42, is required for the chemotactic response to M-CSF but not for locomotion (Allen *et al.*, 1998). This suggests that PI 3-kinase activity may be involved in stimulating Rac and mediating Rho activity and therefore is involved in the re-organisation of the actin cytoskeleton and cell locomotion.

1.7.0. Signal transduction via the M-CSF receptor

C-*fms* has numerous tyrosine phosphorylation sites on its cytoplasmic tail that facilitate its association with various intracellular proteins that mediate M-CSF stimulated responses in cells. Each tyrosine phosphorylation site has the ability to recruit specific cytosolic proteins and protein complexes and it is these interactions and cytosolic proteins, recruited to c-*fms* which will be discussed below. Figure 1.7.1. summaries the c-*fms* tyrosine phosphorylation sites and the proteins that associate with them.

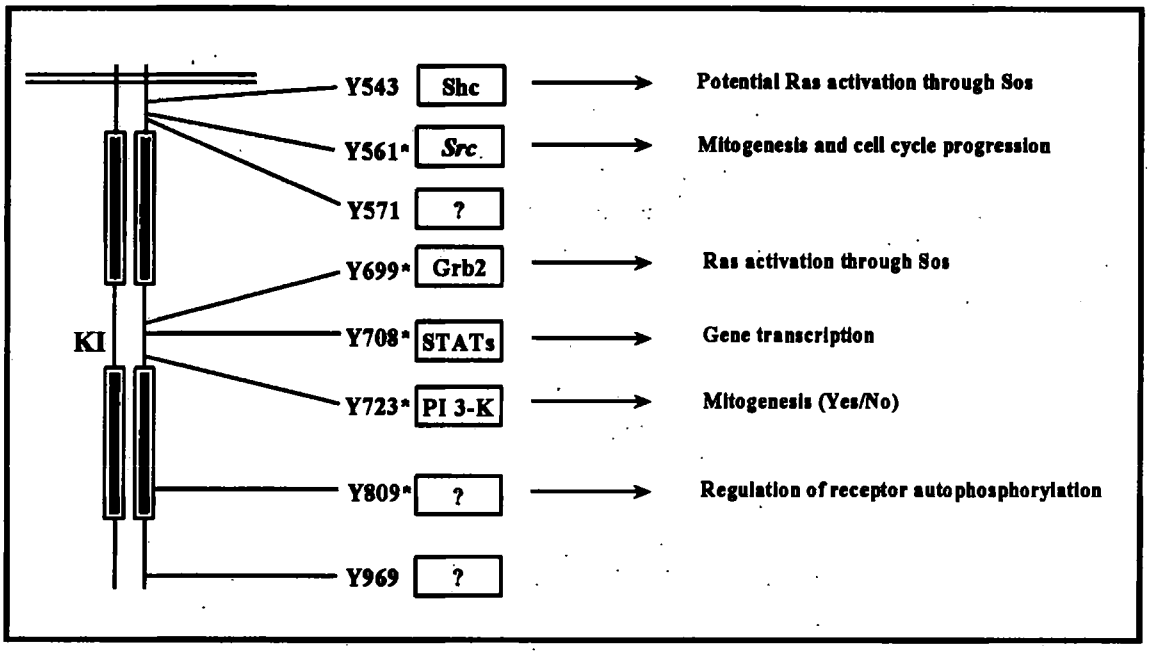


Fig. 1.7.1. Intracellular tyrosine phosphorylation sites on the c-*fms* cytoplasmic tail and cytosolic proteins that associate with them.

1.7.1. The juxtamembrane tyrosine phosphorylation sites

The first 50aa of the cytoplasmic tail, the juxta-membrane region, contains two tyrosine-based activation motifs, at Tyr^{561} and Tyr^{571} and it is possible that a third is present at Tyr^{543} (Fig. 1.7.1.) (Joos *et al.*, 1996; Stanley *et al.*, 1997). Tyr^{561} is the main site of interaction with *src*-family kinases, including *fyn*, *yes* and *src* which are activated as a result of binding to c-*fms* via their SH2 domains (Alonso *et al.*, 1995; Courtneidge *et al.*, 1993). Inhibitory antibodies of *src* family kinases block M-CSF-

stimulated cell-cycle progression from early G_1 phase but are not required prior to commitment to S phase (Roussel, 1994). This suggests that *src*-family kinases are required during the c-*fms* mediated mitogenic signal.

In osteoclasts, M-CSF stimulates cytoskeletal reorganisation, motility and cytoplasmic spreading (Insogna *et al.*, 1997). M-CSF stimulation also increases the detected levels of tyrosine phosphorylation including c-*fms* and *src* and an unidentified 85 kDa protein with a concomitant three-fold increase in *src*-kinase activity (Insogna *et al.*, 1997). In *src*-negative cells, M-CSF fails to induce cell spreading or change F-actin distribution and the 85kDa phosphoprotein was not tyrosine phosphorylated in response to M-CSF stimulation (Insogna *et al.*, 1997). *Src* kinase therefore appears to be required for correct cytoskeletal architecture in the osteoclast.

The second site of tyrosine phosphorylation, Tyr^{571}, also has an SH2 domain recognition sequence and mutation of Tyr^{571} has been shown to inhibit receptor-mediated endocytosis possibly by blocking the c-*fms* tyrosine kinase activity (Carlberg and Rohrschneider, 1994).

A third, putative phosphorylation site at Tyr^{543} has been shown to be phosphorylated in v-*fms* and is required for association of a 55kDa protein (Joos *et al.*, 1996). Tyr^{543} fails to associate with SH2 domain containing proteins, however it appears to be a phosphotyrosine binding (PTB) recognition sequence which binds to Shc, a 55kDa phosphoprotein (Joos *et al.*, 1996). Shc is an adaptor protein that is also found in many recruitment complexes associated with c-*fms* (Rohrschneider *et al.*, 1997). C-*cbl*, a 120kDa protein which is constitutively associated with Grb2 in unstimulated

BAC1.2F5 macrophages, becomes tyrosine phosphorylated within a minute of M-CSF stimulation and is found in a complex with tyrosine phosphorylated Shc and an unidentified 80kDa phosphoprotein (Wang *et al.*, 1996). Simultaneously, phosphorylated c-*cbl* is ubiquitinated and translocated to the plasma membrane and after ten minutes is de-ubiquitinated, de-phosphorylated, and relocalises to the cytosol but remains complexed with Shc for up to one hour (Wang *et al.*, 1996).

1.7.2. Tyrosine residues in the kinase insert domain

One of the defining features of the class III RTK family is an insert in the catalytic domain, the kinase insert domain (KI domain), that disrupts the homology the catalytic domain shares with *src*-family kinases (Carlberg *et al.*, 1991; Roussel *et al.*, 1990). This 72 amino acid insert contains three sites of tyrosine auto-phosphorylation at $Tyr^{699,708,723}$ (Fig. 1.7.1.) (Carlberg *et al.*, 1991; Reedijk *et al.*, 1990; Roussel *et al.*, 1990).

Phosphorylation of Tyr^{699} results in the association of the *Src* Homology 2 (SH2) domain of the adapter molecule Grb2 (Lioubin *et al.*, 1994; van der Geer and Hunter, 1993). Grb2 has been shown to be associated with various other signalling molecules in macrophages including Shc, c-cbl, SHP-1, Sos, and $p150^{SHIP}$ (Chen *et al.*, 1996; Lioubin *et al.*, 1994; Rohrschneider *et al.*, 1997). The association of Grb2 with Sos appears to be constitutive and independent of M-CSF stimulation (Lioubin *et al.*, 1994).

Tyr^{708} is an unusual site of phosphorylation in the c-*fms* because its flanking sequences, which are composed of mostly basic residues, are not homologous to any known tyrosine phosphorylation sites (van der Geer and Hunter, 1990). This hints

towards a more a more specific function than merely a recruitment site for SH2 or PTB domain containing proteins. M-CSF stimulated FDC-P1 cells that express murine c-*fms* exhibit increased STAT-1 and STAT-3 (signal transducers and activators of transcription) activation (Novak *et al.*, 1995; Novak *et al.*, 1996). Activation of STAT-1 requires an intact Tyr^{706} in the murine receptor but this residue does not appear to be required for STAT-3 activation (Novak *et al.*, 1996). In addition, an intact Tyr^{807} in the murine receptor is also required for STAT activation although this residue does not recruit STATs directly (Fig. 1.7.1.) (Novak *et al.*, 1996). The involvement of residue Tyr^{809} will be discussed in more detail later (sections 1.7.3 and 1.7.4.). Tyk-2 , a member of the Jak family kinases, is a possible regulator of STAT activation and is tyrosine phosphorylated after M-CSF stimulation of BMMs. Tyk-2 phosphorylation is also accompanied by Jak-1 phosphorylation in fibroblasts transfected with c-*fms* (Novak *et al.*, 1995). However, phosphorylation of Jak-1 or Jak-2 is not detected in either BMMs or BAC1.2F5s in response to M-CSF which suggests that Tyk-2 phosphorylation is the only regulator of STAT activity in macrophages, or myeloid cells (Novak *et al.*, 1995).

M-CSF stimulates PI 3-kinase activity in murine bone marrow derived macrophages with maximum PI 3-kinase activity observed after ten minutes (Yusoff *et al.*, 1994). The association of PI 3-kinase with c-*fms* was originally mapped using anti-c-*fms* peptide anti-sera which detected a potential PI 3-kinase binding site between residues 701 and 721 in the kinase insert region (Downing *et al.*, 1991). In whole cells only a small minority of ligand activated receptors appear to form a stable complex with PI 3-kinase (Downing *et al.*, 1991) and this interaction was eventually mapped to Tyr^{723} in the KI domain (Reedijk *et al.*, 1992). In BAC1.2F5 cells M-CSF stimulates tyrosine phosphorylation of p85α and its association with c-*fms* and other cytosolic

proteins including c-*cbl*, p95 and p55-60 in a rapid but transient manner (Kanagasundaram *et al.*, 1996). The involvement of PI 3-kinase in c-fms signalling will be discussed in greater detail in sections 1.7.6. and 1.7.7.

In a yeast two hybrid study an autophosphorylated cytoplasmic domain of murine c-*fms* was used to screen an expression library for clones containing SH2 domains which recognised the tyrosine phosphorylation site at Tyr^{721} (Bourette *et al.*, 1997). From this the SH2 domains of PLCγ2 were repeatedly isolated and were shown to interact with Tyr^{721}, directly (Bourette *et al.*, 1997). In c-*fms* expressing FDC-P1 cells M-CSF stimulated the rapid (5minutes→150minutes) but transient phosphorylation of PLCγ2 and this event appeared to be required for activation of the PI 3-kinase pathway (Bourette *et al.*, 1997). It is possible that PI 3-kinase competes with PLCγ2 for the same residue on c-*fms*.

1.7.3. Tyr^{809} in the C-terminal tyrosine kinase domain

The catalytic domain of c-*fms* also contains a kinase lobe with a tyrosine phosphorylation site at Tyr^{809} (Fig. 1.7.1.) (Roussel *et al.*, 1990). This site is common to all receptor tyrosine kinases and on c-*fms*, its auto-phosphorylation is involved in the M-CSF-dependent induction of c-*myc* expression (Fig. 1.7.4.) (Roussel *et al.*, 1991). In studies on the murine receptor a phenylalanine mutation at Tyr^{807} abrogated the differentiation signal but increased the rate of M-CSF-stimulated proliferation in FDC-P1 cells (Bourette *et al.*, 1995). However, mutation of the equivalent residue in the human c-*fms* inhibited M-CSF-induced proliferation in NIH 3T3s with concurrent cell cycle arrest during the early G_1 phase (Courtneidge *et al.*, 1993; Roussel *et al.*, 1990). Additional experiments have shown that antibodies which bind to and

inactivate *src* family kinases also prevent cell cycle progression from early G_1 phase but are not required prior to commitment to S phase (Roussel, 1994).

Mutation of Tyr^{807} in the murine receptor also appears to reduce the c-*fms in vitro* kinase activity (Bourette *et al.*, 1995). Mutating Tyr^{807} to phenylalanine inhibits receptor autophosphorylation suggesting that Tyr^{809} is essential for phosphorylation of other tyrosine residues on c-*fms* (Novak *et al.*, 1996). These mutational studies have underscored the importance of receptor autophosphorylation to recruitment of cytosolic proteins (Reith *et al.*, 1993).

The association of *src*-family kinases with activated c-*fms* also appears to require phosphorylation of Tyr^{809} (Courtneidge *et al.*, 1993). Although there is an increase in *src*-family kinase activity associated with the phosphorylation of Tyr^{809} there is no *src* kinase association, including *fyn* and *yes*, with c-*fms* unless Tyr^{561} is also phosphorylated (Courtneidge *et al.*, 1993). As stated previously, STAT-1 and STAT-3 associate with c-*fms* in BMMs, BAC1.2F5 macrophages and transfected fibroblasts (Novak *et al.*, 1995). Mutation of Tyr^{807} in the murine receptor blocked PLCγ2 binding and phosphorylation but had no affect on the binding or phosphorylation of the p85 subunit of PI 3-kinase (Bourette *et al.*, 1997). The Tyr^{807} mutation also abrogated the differentiation signal in FDC-P1 cells, which is mimicked by PLCγ2 inhibitors.

1.7.4. Tyr^{809} and the immediate early gene response

As previously mentioned the Tyr^{809}Phe mutation abolishes M-CSF-stimulated mitogenesis which is characterised by a significant reduction in c-*myc* expression, although the transcription of immediate early-response genes c-*fos* and c-*jun*B are still

induced (Barone and Courteneidge, 1995; Reedijk *et al.*, 1990; Roussel *et al.*, 1990; Saleh *et al.*, 1995). The importance of c-*myc* expression in c-*fms* signalling is illustrated by demonstration that ectopic c-*myc* expression in NIH 3T3 cells expressing a mutant c-*fms* restores the loss of M-CSF-dependent proliferation (Fig. 1.7.4.) (Barone and Courteneidge, 1995; Downing *et al.*, 1989).

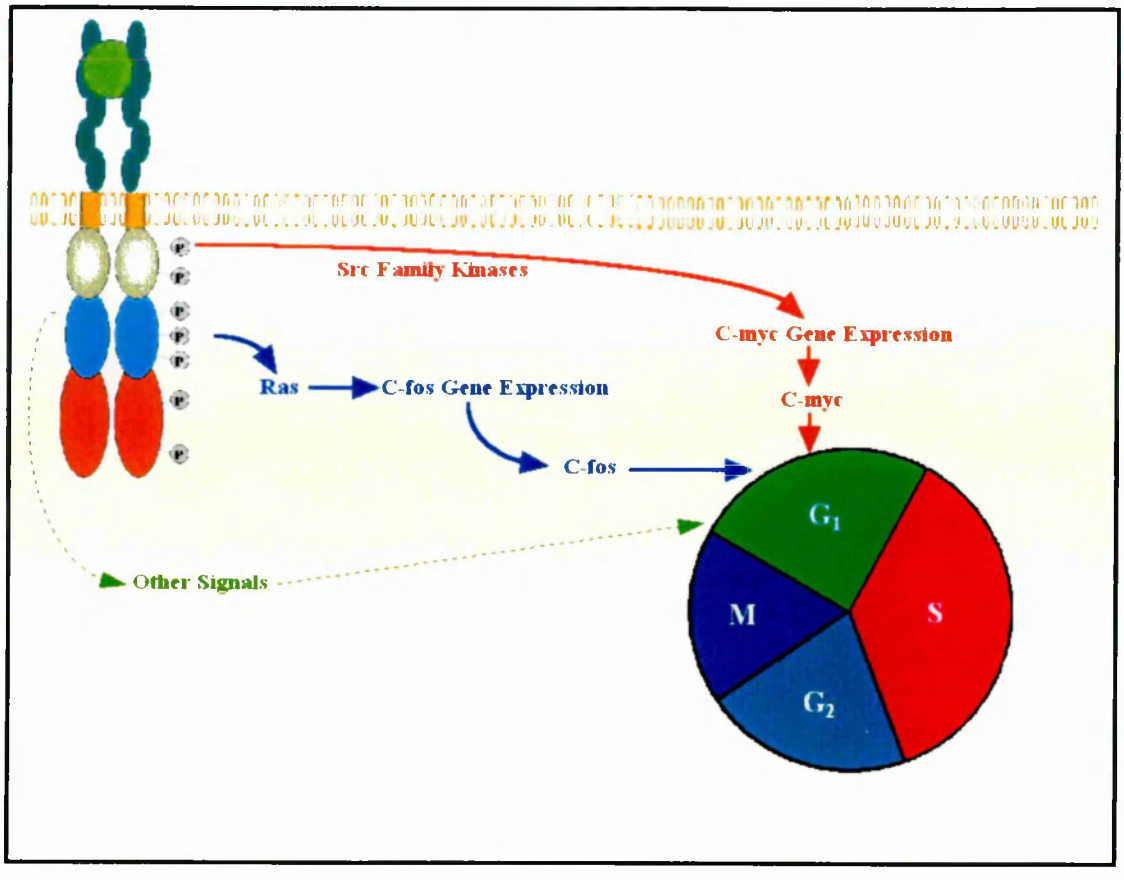


Fig. 1.7.4. **Receptor Stimulation Results in Cell-Cycle Progression.**

In macrophages M-CSF rapidly induces transiently high levels of c-*fos* expression within 15 minutes of stimulation followed by a lower but sustained level of c-*fos* expression for 4-12hrs (Downing *et al.*, 1989). Stimulation of quiescent BAC1.2F5 macrophages with M-CSF results in transient elevation of c-*fos* and subsequent levels of c-*myc* mRNA (Orlofsky and Stanley, 1987).

M-CSF is required by BMMs throughout G_1 for progression into S phase and persistent c-*fms* kinase activity is required not only for the expression of the

immediate early genes (Fig. 1.7.4.), but also for expression of delayed early response genes such as D-type cyclins (Roussel, 1997). Mutational analysis has shown that Tyr^{561} and Tyr^{809} are required for induction of c-*myc* expression, and Tyr^{571}, Tyr^{699}, Tyr^{708} and Tyr^{723} are required for c-fos and c-jun expression (Roussel, 1997). This evidence suggests that the c-*fms* mitogenic signal occurs via two pathways. One pathway which leads to transcription/translation of c-*fos* and c-*jun*B and is independent of the phosphorylation state of Tyr^{809} but involves tyrosine residues in the KI domain, and a second pathway which is phosphorylation-dependent and leads to c-*myc* transcription which requires *src* kinase activity.

1.7.5. The oncogenic receptor: V-*fms*

The v-*fms* homologue of c-*fms* was originally identified as the oncogene carried by the SM and HZ5 strains of feline sarcoma virus, FeSV, (Genbank accession number K01643) (Hampe *et al.*, 1984). Feline v-*fms* differs from the feline c-*fms* by 12 amino acid substitutions distributed throughout the protein and by the replacement of a 50aa sequence close to the carboxyl-terminal tail region by 14 unrelated amino acids (Sherr and Stanley, 1990). The carboxyl-terminal tail region of c-*fms* is 24aa in length and appears to function as a negative regulator of receptor tyrosine kinase activity, controlled to some degree by phosphorylation at Tyr^{969} (van der Geer and Hunter, 1990). Modification of this tail region resulting in the loss of Tyr^{969} prevents regulation of receptor autophosphorylation, implying that Tyr^{969} is involved in the negative regulation of receptor tyrosine kinase activity (Sherr and Stanley, 1990). The carboxyl-terminal deletion in conjunction with two point mutations present in the D_4 loop at positions 301 and 374, abolishes the dependence on M-CSF for initiation of receptor signalling (Sherr and Stanley, 1990). Mutation of Leu^{301} in the extracellular domain, in the D_4 loop, has also been shown to be sufficient for

activation of the c-*fms* transforming potential (Roussel *et al.*, 1988). Cells transformed by v-*fms* also exhibit increased levels of PI turnover with conversion of $PI_{4,5}P_2$ to inositol trisphosphate (IP_3) to mobilise Ca^{2+} and Diacylglycerol (DAG) to activate Protein Kinase C (PKC). This is associated with the increased activities of membrane associated $PI_{4,5}P_2$-PLC and PI kinase activity which implies there is a role for $PI_{4,5}P_2$-derived secondary messengers in c-*fms* signalling (Whetton *et al.*, 1986).

A second, potential Grb2 binding site has been mapped on oncogenic v-*fms* at Tyr^{921} which appears to bind to Grb2 with higher affinity than Tyr^{699} (Mancini *et al.*, 1997). NIH 3T3 cells expressing v-*fms* containing a mutation at Tyr^{807} have an increased fibronectin network and have lost serum-independent growth typically observed in wild type v-*fms* transformed NIH 3T3 cells (Mancini *et al.*, 1997). Further investigation of this phosphorylation site may reveal alternative mechanisms for cell transformation.

Mutational analysis has shown that phosphorylation of Tyr^{807} on v-*fms* controls cell morphology and the association of p120Ras•GAP in transformed NIH 3T3 cells, although mitogenesis was not blocked by this mutation (Trouliaris *et al.*, 1995). However this conflicts with earlier reports on the effects of mutation at Tyr^{809} of c-*fms* expressed in fibroblasts (Roussel *et al.*, 1990), therefore it is possible that the physical differences between v-*fms* and c-*fms* also affects how mitogenic signals are transduced. Additionally, phosphorylation of p120Ras•GAP does not occur in BAC1.2F5 macrophages (Reedijk *et al.*, 1990). Thus from studies of v-*fms*, Tyr^{807} and the equivalent residue on the murine and human receptors, appears to regulate the intrinsic tyrosine kinase properties of c-*fms* and the actin cytoskeleton via Ras•GAP. The association of the Ras•GAP with v-*fms* and its apparent non-involvement in v-

fms mediated mitogenesis is something of a paradox. However, it is possible that other sites are involved in Ras activation or that the cell type in which a mutated c-*fms* is expressed also determines the biological outcome of the mutation. There are data that seem to indicate that cell type influences the results obtained with respect to PI 3-kinase and must therefore be considered during their interpretation and this will be discussed below.

1.7.6. Evidence for the involvement of PI 3-kinase in c-*fms* signalling

Expression studies in NIH 3T3 cells with the PDGFr mutated at the binding sites for PI 3-kinase and either Ras•GAP, SHP-2 or PLCγ have shown that binding of PI 3-kinase to tyrosine phosphorylated PDGFr was necessary for full PI 3-kinase activity (Klinghoffer *et al.*, 1996). In addition, accumulation of GTP-Ras was also required for full PI 3-kinase activition (Klinghoffer *et al.*, 1996).

In corollary experiments performed in transfected rat fibroblasts (208F) a mutation at Tyr^{721} in murine c-*fms* resulted in a marked decrease in the ability of c-*fms* to bind the SH2 domain of the p85 subunit of PI 3-kinase with a concomitant loss in PI 3-kinase activity (Reedijk *et al.*, 1992; van der Geer and Hunter, 1993). Metabolic labelling of BAC1.2F5 macrophages suggested only a minor PI 3-kinase activity in c-*fms* immunoprecipitates but indicates that they exist as a stable complex since the interaction between p85α and the tyrosine phosphorylated proteins survived anion exchange chromatography (Kanagasundaram *et al.*, 1996).

M-CSF stimulation of a stable transfectant CHO cell line expressing human c-*fms* induces sustained tyrosine phosphorylation of c-*cbl* and its subsequent association with Crk-II, and the p85 subunit of PI 3-kinase (Husson *et al.*, 1997). In this study it

appears that p85 exists as a constitutively associated complex with Grb2 (Husson *et al.*, 1997). Both p85 SH2 domains are required for this complex formation and it appears that in this cell model c-*cbl* immunoprecipitates contain the major fraction of PI 3-kinase activity in c-*fms* transformed CHO cells stimulated with M-CSF (Husson *et al.*, 1997).

As described earlier PLCγ2 can associate with Tyr^{723} of c-*fms*. In addition, mutation of Tyr^{807} abrogates the c-*fms*-mediated differentiation signal in FDC-P1 cells, which is mimicked by PLCγ2 inhibitors (Bourette *et al.*, 1997). However, it is also possible that PI 3-kinase activates PLCγ2 via production of $PI_{3,4,5}P_3$ from their shared substrate, $PI_{4,5}P_2$. It has been shown that $PI_{3,4,5}P_3$ activates PLCγ2 *in vitro* by interfering with its SH2 domains. Therefore it is more likely that Tyr^{723} associates with PI 3-kinase in preference to PLCγ2 because Tyr^{723} contains a classical YXXM motif which has been shown to associate with the p85 SH2 domain with high affinity and it has been demonstrated that the PLCγ binding site on the PDGFr binds the p85 SH2 domain with 30-40 fold lower affinity than that for PLCγ (Piccione *et al.*, 1993). Therefore *in vitro* associations may not be truly representative of physiological interactions and it is more likely that the observed activation of PLCγ2 occurs sequentially after PI 3-kinase activation in myeloid cells.

As for a role for PI 3-kinase in the proliferative response, early data suggested that mutation of Tyr^{721} in the murine receptor when expressed in Rat-2 fibroblasts, or expression in NIH 3T3 cells of a human c-*fms* lacking the entire KI domain resulted in a concomitant loss of PI 3-kinase activity and cell proliferation (Reedijk *et al.*, 1992; van der Geer and Hunter, 1993). However, there is a paucity of data on the involvement of PI 3-kinase in other M-CSF-stimulated cell responses, but it has been

observed that in macrophages, PI 3-kinase inhibitors block endocytosis not by interfering with the initiation of the process but rather by preventing its completion (Araki *et al.*, 1996). What is apparent from this study is that wortmannin had a greater affect on macropinocytosis than micropinocytosis. Since macropinocytosis is an actin-mediated process, it is possible that the effects of wortmannin on macropinocytosis in macrophages may not indicate a direct requirement of PI 3-kinase activity in vesicle integrity or formation but rather in the correct reorganisation of the actin cytoskeleton on which macropinocytosis relies (Araki *et al.*, 1996).

1.7.7. Evidence against PI 3-kinase involvement in c-*fms* signalling

It has been demonstrated in c-*fms* transfected NIH 3T3 cells that deletion of the KI domain only slightly impaired M-CSF induced cell proliferation, suggesting that tyrosine residues involved in PI 3-kinase and Grb2 binding alone are not essential for the mitogenic signal (Shurtleff *et al.*, 1990). Furthermore, in FDC-P1 cells transfected with a c-*fms* mutant lacking the entire KI domain, M-CSF still stimulated cell proliferation at levels similar to wild type c-*fms* transfected cells (Kanagasundaram *et al.*, 1996). These data seem to suggest that PI 3-kinase activity, after all, is not required during M-CSF stimulated cell proliferation.

Grb2•Sos activation may be required during growth signals of c-*fms* expressed in fibroblasts, this seems to be only a minor or alternative pathway in myeloid cells (Lioubin *et al.*, 1994). Recently in a review, J. Hamilton claimed to have unpublished data which suggested that PI 3-kinase activity, when inhibited by wortmannin, was not required for M-CSF stimulated DNA synthesis in bone marrow derived macrophages (Hamilton, 1997). In studies of murine c-*fms* expressed in the FDC-P1 cell line, mutations at Tyr^{697}, Tyr^{706} and Tyr^{721} did not prevent cell differentiation, but

intriguingly seemed to augment this M-CSF stimulated process (Bourette *et al.*, 1995).

1.8.0. Further analysis of PI 3-kinase in c-*fms* signalling

It is apparent that further analysis of c-*fms* signalling pathways is required before a clear understanding of its biology is achieved. Various gaps exist in relation to the activation of PI 3-kinase downstream of c-*fms* and it is the intention of this thesis to try to address some of these issues. Firstly, the existing data is inconsistent and can be interpreted differently, depending on various parameters including the cells in which c-*fms* is expressed, the various approaches to mutational analysis employed and even the species of receptor investigated. It is impossible to address all these issues in one study, however the most important aspect to consider in resolution of these discrepancies must lie in the model cell system used for investigation.

To date, a large proportion of c-*fms* analysis has occurred in transfected fibroblasts or epithelial cells which, although a suitable model for other RTKs such as the PDGFr, may not mimic closely enough the relevant cellular background in which c-*fms* is normally found. A more suitable model for investigation of c-*fms* signalling would be a cell line that normally expresses c-*fms*, but would also provide new insights into macrophage function. One such cell line is BAC1.2F5, a macrophage-like adherent cell line that is dependent on M-CSF for survival and proliferation (Morgan *et al.*, 1987). This cell line has been characterised for its morphological and proliferative responses to M-CSF and has revealed some interesting results. In BAC1.2F5 cells M-CSF stimulation has been reported to stimulate the phosphorylation and activation of various cytosolic proteins including; Rho family GTPases which are involved in regulation of the actin cytoskeleton (Allen *et al.*, 1997); the transcription factors

STAT-1 and STAT-3; the formation of a complex contain Shc, c-*cbl* and Grb2 (Novak *et al.*, 1995) and SHP-1 which contains tyrosine phosphatase activity (Yeung *et al.*, 1992). In addition, through *in vitro* studies of BAC1.2F5 cells and analysis of other macrophage cell models it has been demonstrated that *src*-family kinases (Li *et al.*, 1997) and PI 3-kinase (Kanagasundaram *et al.*, 1996) also associate with activated c-*fms*. The mapping of available binding sites on c-*fms* has allowed various models of c-*fms* signalling to be proposed, however it is difficult to ratify all the published observations based on this limited data. Therefore it is necessary to further explore the function of PI 3-kinase activation during c-*fms* signalling. To do this a cellular and molecular approach has been employed throughout this study.

Chapter 2

Aims of Thesis

2.0.0. Aims of thesis

The initial aim of this thesis was to characterise the involvement of PI 3-kinase in the various M-CSF stimulated responses observed in BAC1.2F5 macrophages. Expression of c-*fms* in BAC1.2F5 cells was initially characterised by biochemical means, as well as expression of various cytosolic components that have been reported to be involved in c-*fms* signalling in BAC1.2F5 and other cell systems. It was also important to identify the PI 3-kinase subunit isoforms present and activated in BAC1.2F5 cells since only classical p85α/p110α have been reported in c-*fms* signalling and discovery of other PI 3-kinase subunits would confer alternative mechanisms for phospholipid synthesis.

Requirements for PI 3-kinase activity in vesicle trafficking and the morphological changes observed in BAC1.2F5 cells was characterised using PI 3-kinase-specific inhibitors, wortmannin and LY294002. At concentrations at or near their respective IC_{50} both inhibitors specifically inhibit D3 phosphoinositide synthesis, but not the activity of PI-specific 3-kinase. Trafficking of internalised receptor was investigated by immunofluorescent microscopy and PI 3-kinase inhibitors were employed to identify whether PI 3-kinase activity is required for receptor trafficking after internalisation of c-*fms*. In addition it has previously been reported that Rac is a downstream mediator of PI 3-kinase activity (Bokoch et al., 1996) and therefore may be involved in the regulation of the actin cytoskeleton. Thus the requirement of PI 3-kinase activity in the actin-mediated morphological changes observed by Allen et al, 1997 was also investigated using the PI 3-kinase inhibitor, LY294002.

PI 3-kinase inhibitors were also used to investigate PI 3-kinase function in cell proliferation and survival in an attempt to reconcile the previously published

conflicting data. The role of PI 3-kinase in M-CSF stimulation of DNA synthesis and cell proliferation was investigated using non-radioactive BrdU incorporation assays. The M-CSF stimulated survival of BAC1.2F5 cells was also investigated using PI 3-kinase inhibitors and this was characterised by an assay for apoptosis, since PI 3-kinase has been shown to activate PKB, which contributes to protection against apoptosis in various cell models.

The specificity of PI 3-kinase inhibitors when used at their IC_{50} concentrations makes them extremely useful tools for characterising PI 3-kinase activation downstream of c-*fms*. However it is not possible to investigate how PI 3-kinase interacts with c-*fms* whether direct or indirect. It has been shown that PI 3-kinase associates directly with phosphorylated c-*fms* via Tyr^{723} and this interaction may also involve an additional tyrosine phosphorylated residue, at Tyr^{708}. In addition PI 3-kinase has also been found in a complex with Grb2•Sos (Saleem et al., 1995) and the potential to form this complex exists in BAC1.2F5 cells providing an indirect mechanism for PI 3-kinase activation.

Analysis of PI 3-kinase association with tyrosine phosphorylated c-*fms*, was investigated with a series of expression constructs based on human c-*fms* that harbour amino acid substitutions at Tyr^{723} and Tyr^{708} mutated either alone or in tandem. Construction of these expression constructs also involved introducing C-terminus epitope tags to facilitate identification and purification of receptor complexes. Over-expression of these receptor constructs in BAC1.2F5 cells was intended to compete for M-CSF with endogenous receptor and therefore inhibit PI 3-kinase activation in a dominant/negative manner.

Chapter 3

Materials and Methods

3.1.0. Reagents and equipment

3.1.1. Cell Culture

RPMI medium 1640 (Gibco-BRL, Paisley, Scotland)

DMEM medium (Gibco-BRL)

Foetal Bovine Serum, heat inactivated, (Gibco-BRL)

Trypsin-EDTA (Imperial Laboratories, Hampshire, England)

Accutase (Innovative Cell Technologies, California, USA)

L-Glutamine 200mM (Gibco-BRL)

HEPES buffer 1M (Gibco-BRL)

Penicillin/Streptomycin solution (Gibco-BRL)

3.1.2. Cell Lines

A431 Human epithelial carcinoma (ECACC, #85090402)

NIH 3T3 Murine fibroblast (ECACC, #93061524)

U937 Human histiocytic lymphoma CRL-1593 (ATCC)

HEK 293 Human transformed primary embryonic kidney CRL-1573 (ATCC)

BAC1 Murine macrophage (G.E. Jones, Randall Inst. London, England)

BAC1.2F5 Murine macrophage, subclone of BAC1 cell line (E.R. Stanley, AECOM, New York, USA)

3.1.3. Microscopy

Microscope slides 1.0-1.2mm (BDH Laboratory Supplies, Leics., England)

Circular 13mm glass coverslips No.1 (BDH)

FluoroGuard™ anti-fade reagent, (Bio-Rad Laboratories, California, USA)

Axiovert 135 inverted microscope, x40, x63 and X-100 (Zeiss,

Axiovert 35 inverted microscope, x32 (Zeiss)

Axioplan inverted microscope, x10, x20, x40, x63 and X-100 (Zeiss)

3.1.4. Primary Antibodies

c-*fms* Rat mAb clone 3-4A4 human reactive, (Santa Cruz Biotechnology, CA, USA)

c-*fms* Rabbit pAb clone C-20 human and murine reactive, (Santa Cruz)

c-*fms* Rabbit pAb human reactive, (Upstate Biotechnology, NY, USA)

c-*fms* Rat mAb clone AFS-10-6-1 human reactive, (Pharmingen, CA, USA)

c-*fms* Rat mAb clone 2-4A5 human reactive, (Santa Cruz)

p110α Rabbit pAb human reactive, (Santa Cruz)

p110α Rabbit pAb human and murine reactive, (Transduction Labs., KY, USA)

p110β Rabbit pAb human and murine reactive, (Santa Cruz)

p110γ Rabbit pAb human and murine reactive, (Santa Cruz)

p85α Murine mAb human and murine reactive, (Transduction Laboratories)

p85α-Protein A/Agarose Rabbit pAb human and murine reactive, (Upstate Biotech.)

p85β Murine mAb human and murine reactive, (Serotec Ltd., Oxford, England)

p85γ Murine mAb clone V-2 human and murine reactive, (Serotec Ltd)

SHIP Goat pAb clone M-14 murine reactive, (Santa Cruz)

Shc Rabbit pAb human and murine reactive, (Transduction Laboratories)

Grb2 Murine mAb clone 81 human and murine reactive, (Transduction Laboratories)

Sos1 Murine mAb clone 25 human and murine reactive, (Transduction Laboratories)

Phosphotyrosine Murine mAb human and murine reactive, (Upstate Biotech.)

c-*src* Rabbit pAb human and murine reactive, (Santa Cruz)

c-*myc* Murine mAb clone 9E10 human reactive, (Calbiochem, Notts, England)

c-*cbl* Rabbit pAb human and murine reactive, (Santa Cruz)

3.1.5. Secondary Antibodies

HRP-conjugated Goat anti-mouse IgG pAb, (Bio-Rad)

HRP-conjugated Goat anti-rabbit IgG pAb, (Bio-Rad)

Alexa Dye 488-conjugated Goat anti-mouse IgG pAb, (Molecular Probes, OR, USA)

Alexa Dye 488-conjugated Goat anti-rabbit IgG pAb, (Molecular Probes)

Alexa Dye 488-conjugated Goat anti-rat IgG pAb, (Molecular Probes)

Alexa Dye 594-conjugated Goat anti-mouse IgG pAb, (Molecular Probes)

Alexa Dye 594-conjugated Goat anti-rabbit IgG pAb, (Molecular Probes)

Alexa Dye 594-conjugated Goat anti-rat IgG pAb, (Molecular Probes)

FITC-conjugated Sheep anti-mouse IgG pAb, (Amersham Pharmacia Biotech., Bucks, England)

FITC-conjugated Donkey anti-rabbit IgG pAb, (Amersham)

Texas Red-conjugated Sheep anti-mouse IgG pAb, (Amersham)

Texas Red-conjugated Donkey anti-rabbit IgG pAb, (Amersham)

FITC-conjugated Goat anti-rat IgG pAb, (Caltag Laboratories, CA, USA)

3.1.6. General Reagents

0.5M EDTA (pH8.0) (Sigma Chemical Co., Dorset, England)

0.5M Tris.HCl (pH6.8) (Sigma)

1.0M Tris.HCl (pH6.8) (Sigma)

1.5M Tris.HCl (pH8.8) (Sigma)

10mM Tris.HCL (pH7.2) (Sigma)

100mM I.P.T.G. (Isopropyl-D-thiogalactopyranoside, Sigma) in deionised (d.i.) water

10% Ammonium persulphate solution (BDH) in d.i. water

10% (w/v) SDS (Sigma) in d.i. water

1X phosphate buffered saline (PBS_A) (Oxoid, Hamps., England) 1 tablet/100ml d.i. water

2.5mg ml^{-1} Ethidium Bromide (Sigma)

2.5M NaCl (BDH)

3M Sodium acetate (pH4.6) (Sigma)

4M Ammonia solution (Sigma)

40% Formaldehyde solution (BDH)

40mg ml^{-1} X-gal (5-Br-4-Cl-3-indolyl-D-galactoside) (Sigma) in dimethylformamide

5M NaCl (BDH)

50mg ml^{-1} carbenicillin (Sigma) made up in d.i. water

50mM Glycine buffer (pH2.0) (BDH)

50mM HEPES (pH7.5) (Gibco-BRL)

70% (v/v) and 95% (v/v) ethanol (Fisher Sci., Leics., England) made up in d.i. water

Acetic acid (BDH)

Agar N°. 1 (Lab M, Lancs., England)

Agarose, electrophoresis grade (Gibco-BRL)

Bacto-tryptone (Difco laboratory supplies, MI, USA)

Bacto-yeast extract (Difco)

β-Mercaptoethanol (Sigma)

Bis acrylamide 30% (w/v) (37.5:1) (Bio-Rad)

Bi-tek agar (Difco)

Boric acid (BDH)

Bovine serum albumin (BSA) powder (Sigma)

Bromophenol Blue (Sigma)

Butanol (Fisher Scientific)

Chloroform (BDH)

Citifluor (UKC, Canterbury, England)

Coomassie blue (BDH)

d.i. water

DEPC treated d.i. water

Diethyl pyrocarbonate (DEPC) (BDH)

Dimethylformamide (Sigma)

Ethanol (Fisons Scientific Equipment, Leics., England)

Fibronectin (Sigma)

Glycerol (BDH)

Glycine (BDH)

Industrial methylated spirits (IMS) (Fisher Scientific)

Marvel (Premier beverages, Staffs, England)

Methanol (Fisons)

mineral oil (Sigma)

Nail polish (Boots the Chemists, Notts, England)

Nonidet NP-40 (Fisons)

Phorbol 12-Myristate 13-Acetate (PMA) (Sigma)

Polyethylene glycol (PEG) (Sigma)

Potassium oxalate (Sigma)

Propan-2-ol (Fisher Scientific)

S.O.C. medium (Gibco-BRL)

Sodium dodecyl sulphate (SDS) (Sigma)

Sodium orthovanadate (Sigma)

TEMED (Sigma)

Tris Base (Sigma)

Triton X100 (Sigma)

Tween 20 (polyethylene sorbitan monolaurate) (Sigma)

3.1.7. General Solutions

Coomassie Stain

- Coomassie blue	0.1% (w/v)
- IMS	50.0% (v/v)
- Acetic acid	10.0% (v/v)

Made to 1 litre with d.i. water

Slow de-stain solution

- Methanol	20.0% (v/v)
- Acetic acid	7.1% (v/v)

Made up to 1 litre in IMS

SDS-PAGE Sample buffer (x2):

- Glycerol	11.2% (w/v)
- 1M Tris (pH6.8)	8.9% (v/v)
- SDS	1.1% (w/v)
- β-Mercaptoethanol	5.0% (v/v)
- Bromophenol Blue (0.2% stock solution)	1.0% (w/v)

Made up to 100ml with d.i. water

SDS-PAGE running buffer (x10):

- Tris base	0.25M
- Glycine	1.92M
- SDS	1.0% (w/v)

Made up to 1 litre with d.i. water.
Stored at room temperature and diluted 1/10 before use.

Semi-dry transfer buffer (pH9.2):

- Tris base	48mM
- Glycine	39mM
- Methanol	20% (v/v)

Made up to 1 litre with d.i. water

PI 3-kinase buffer (2X)

- Tris Base	40mM
- NaCl	200mM
- EGTA	1mM

Lysis Buffer

- Nonidet NP-40	1% (v/v)	
- Sodium chloride	0.1M	
- Tris.HCL pH7.2	10mM	
- EDTA	1mM	
- Sodium σ-vanadate*	100μM	*Optional.
- Protease inhibitor cocktail (Sigma)	100μl per $1x10^7$ cells	

X10 TBE buffer (pH8.3)

- Tris base	0.89M
- Boric acid	0.89M
- 0.5M EDTA (pH8.0)	4% (v/v)

X10 DNA electrophioresis loading buffer

- Ficoll 400	20% (v/v)
- EDTA pH8.0	0.1M
- SDS	1.0% (w/v)
- Bromphenol blue	0.25% (w/v)
- Xylene Cyanol	0.25% (w/v)

L.B. broth

- Bacto-tryptone	1.0% (w/v)
- Bacto-yeast extract	0.5% (w/v)
- Sodium chloride	0.5% (w/v)

Made up to 1 litre with d.i. water

L.B. Agar

- Agar N°. 1	1.5% (w/v) in L.B. broth

3.1.8. General Equipment

J2-MC centrifuge (Beckman Instruments Inc., CA, USA)

GS-6R bench-top centrifuge (Beckman)

RC-5C centrifuge (Sorvall, Herts., England)

Mini-PROTEAN II electrophoresis cell (BioRad)

Model 200 power supply (BioRad)

Eppendorf 5402 refrigerated microcentrifuge (Fisher Scientific)

Eppendorf 5417C microcentrifuge (Eppendorf)

Dri-block DB-1 (Techne, Cambs., England)

WhirliMixer (Nickel Electrical Ltd., Avon, England)

PS500XT power supply (Amersham Pharmacia Biotech Ltd., Bucks., England)

FACScan (Beckton Dickinson, Oxford, England)

Sonifier 250 (Branson Ultrasonics Corporation, CT, USA)

DNA speedvac DNA100 (Savant Instruments Inc., NY, USA)

Packard InstantImager™ (Canberra Packard Ltd. Berks, England)

TransBlot SD semi-dry transfer cell (BioRad)

Belly dancer (Stovall Life Science, NC, USA)

Rotator (Stuart Scientific, Surrey, England)

Curix 60 automatic developer (AGFA-Gevaert Ltd., Middx., England)

Compact-4 automatic developer (X-Ograph Ltd., Gloucs., England)

Axioplan microscope (Zeiss)

Axiovert 35 microscope (Zeiss)

Axiovert 135 microscope (Zeiss)

OpenLab 3.0 image acquisition system (Improvision, Coventry, England)

FX-35DX 35mm SLR camera (Nikon UK Ltd., Surrey, England)

UFX-DC camera controller (Nikon)

Horizon 11.14 mini-gel DNA electrophoresis system (Gibco-BRL)

Horizon 58 midi-gel DNA electrophoresis system (Gibco-BRL)

Omnigene DNA thermocycler (Hybaid,)

PH-3 DNA thermocycler (Techne)

37°C incubator, humidified and flushed with 5% CO_2 (Jencons Scientific Inc., Beds., England)

3.1.9. General consumables

Tissue culture flasks, 25mm^2, 75mm^2 and 175mm^2, (Nalge, Hereford, England)

Surface™ tissue culture 6, 24 and 96-well plates (Nalge)

30ml Universal centrifuge tubes (Bibby Sterilin Ltd., Staffs., England)

50ml polypropylene centrifuge tubes (Falcon)

Hybond™ ECL nitrocellulose membrane (Amersham)

Hyperfilm™ ECL autoradiographic film (Amersham)

Hyperfilm™ cassette (Amersham)

10cm^2 Square petri dishes (Bibby Sterilin Ltd.)

Thermowell thin-wall 96-well plate model H (Costar)

30mm, 90mm and 140mm Petri dishes (Bibby Sterilin Ltd.)

14ml centrifuge tubes (Falcon)

Microbiological plate spreader (L.I.P.)

Innoculation loops (Starstedt)

60Å Silica gel thin layer chromatography (TLC) plates (Whatman)

1ml, 2ml, 10ml, 20ml sterile syringes (Beckton Dickinson)

Microlance 25g needles (Beckton Dickinson)

3.2.0. Cell biology

3.2.1. Cell Culture

All reagents were supplied by Gibco-BRL unless otherwise stated. The suspension cell lines U937 and J774 were maintained in growth medium consisting of RPMI supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine, 50μg ml^{-1} streptomycin and 50 units ml^{-1} penicillin. The adherent Bac1, Bac1.2F5, HEK 293 and Cos-7 cells lines were maintained in growth medium consisting of DMEM supplemented with 10% (v/v) foetal calf serum, 2mM L-glutamine, 50μG ml^{-1} streptomycin and 50 units ml^{-1} penicillin. Bac1.2F5 cells were supplemented with 10ng ml^{-1} recombinant murine M-CSF (R&D Systems). Cells were grown at 37°C, 5% CO_2 in T75 tissue culture flasks (Nuncleon). Cells were subcultured as required by dissociation with either 0.25% Trypsin/ 0.02% EDTA in phosphate buffered saline (Imperial Labs.) or Accutase (Innovative Tech. Inc.), and resuspended in growth medium at approximately 2 x 10^5 cells ml^{-1}. Quiescent Bac1.2F5 macrophages were obtained by removing M-CSF from the culture medium. Quiescent Bac1.2F5 macrophages were re-stimulated by addition of 50 ng ml^{-1} M-CSF after 24 hours in the absence of M-CSF.

3.2.2. Time lapse photomicroscopy

Bac1.2F5 cells were seeded at 1x10^5 cells per well of a 6-well tissue culture plate containing four 13mm round cover slips per well. At approximately 80% confluency cells were quiesced for at least 24 hours in normal medium in the absence of M-CSF growth factor. Single coverslips were removed to a 35mm petri dish and washed

once with PBS_A. PBS_A was aspirated immediately and replaced with normal medium containing 10mM HEPES. The petri dish was placed onto a Zeiss Axiovert 130 inverted microscope. Normal culture temperature of 37°C was maintained by localised heating of the air around the petri dish with a small electric room heater. Growth factor and inhibitors were added drop-wise from a Pasteur pipette without disturbing the coverslip. The experiments were documented with a Nikon FX-35DX 35mm camera controlled by a UFX-DC camera controller.

3.3.0 Protein biochemisty

3.3.1. Western blot analysis

Protein isolation:

Cultured, adherent cell lines were detached from tissue culture vessels with either Trypsin/EDTA or Accutase. The protease activity was neutralised by addition of fresh culture medium containing 10% FCS and cell pellets were obtained by centrifugation at 1,500 rpm for 8 minutes. Pellets were washed once with PBS_A and total cellular protein was solubilised by addition of 2x SDS sample buffer. Cells routinely cultured in suspension were pelleted by centrifugation at 1,500rpm for 8 minutes. Pellets were washed once with PBS_A and total cellular protein was solubilised by addition of 2x SDS sample buffer. Approximately $1x10^6$ cells were solubilised in 100µl of 2x SDS sample buffer. All protein samples were drawn 5-6 times through a 23-gauge needle to disperse protein aggregates and to shear DNA,

heated to 100°C for 3 minutes, centrifuged at 14,000rpm for 5 minutes and placed on ice prior to loading.

Electrophoretic separation of proteins:

Protein separation was carried out by one-dimensional discontinuous (Laemmli) gel electro-phoresis under denaturing conditions (Laemmli, 1970). All protein gels were carried out on a Mini-PROTEAN II electrophoresis cell (Biorad). Protein gels were cast as follows;

1. Glass plates were washed thoroughly with d.i. water and air-dried. Plates were wiped with a tissue soaked in IMS and left to air dry. Assembly of glass plate sandwich was carried out following Biorad protocol and the sandwich was locked into the casting stand.

2. The resolving gel was prepared for each required gel density as in Table X. Approximately 4ml of resolving gel solution was added to each glass plate sandwich and 500µl of d.i. water-saturated butanol was overlayed to prevent evaporation from the resolving gel during polymerisation. Resolving gels were allowed to polymerise for 30 minutes.

3. Butanol was removed by washing with copious amounts of d.i. water. Excess water was removed by careful drying with a tissue. The stacking gel was prepared as in Table 3.3.1..

4. A 1mm 15 tooth Teflon comb was inserted into the glass plate sandwich before addition of the stacking gel. Stacking gel was carefully added to prevent introduction

of air bubbles until the gel completely filled the spaces and the height of the gel solution in the sandwich reached the top of the small glass plate. Stacking gels were allowed to polymerise for 30 minutes.

5. The glass plate gel sandwich was carefully removed from the casting rack and attached to the inner-cooling core of the gel running apparatus. This assembly was then placed in the gel tank.

6. The gel tank was half filled with 1x SDS electrophoresis running buffer and any air bubbles on the bottom edge of the acrylamide gel were dispersed by agitation. The inner chamber was then filled with 1x SDS electrophoresis running buffer until the inner glass plate and comb were submerged. The Teflon casting comb was then carefully removed.

7. The required samples and markers were loaded onto the gel and empty wells were filled with 10µl of 1x SDS sample buffer. The upper and lower chambers were filled with 1x SDS electrophoresis buffer and the lid was assembled. The power supply was connected and a constant voltage of 150 volts was maintained until the protein samples had migrated the required distance.

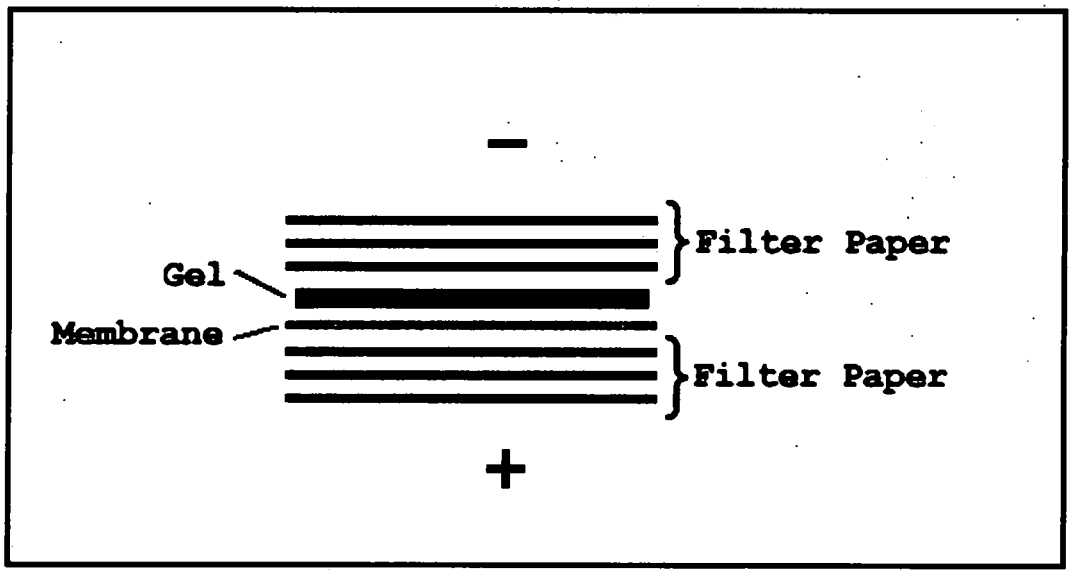
8. The gel apparatus was carefully disassembled and the gels were removed from the glass plates, discarding the stacking gel, and placed in 1x SDS electrophoresis buffer prior to immunoblotting to membrane.

Immunoblotting:

Immunoblotting was carried out by electrophoretic transfer from the gel to a Hybond ECL nitrocellulose membrane, (Amersham) for subsequent analysis. Blotting was carried out in a semi-dry transfer apparatus as follows;

1. For transfer of one gel, six sheets of Whatmann filter paper and one sheet of Hybond ECL cut just larger than the gel being transferred were pre-soaked in semi-dry transfer buffer for 10 minutes. Each gel was pre-soaked in semi-dry transfer buffer for 10 minutes.

2. Each stack of filter paper, membrane and gel were assembled on the bottom electrode of the transfer tank as follows insuring that no air bubbles were introduced;



3. The transfer tank was assembled and the electrodes were attached to a power supply. A constant voltage of 18 volts for two gels was applied for 45 minutes.

5. The transfer tank was disassembled and the nitrocellulose membrane was removed and placed in PBS_A/0.1% Tween prior to probing. To demonstrate efficient transfer of proteins, the acrylamide gel was placed in Coomassie stain for 2-3 hours to stain

protein bands. The acrylamide gels were washed overnight in "slow" de-stain solution to visualise protein bands which had not been completely transferred.

Immunoprobing:

Proteins separated by SDS-PAGE and immobilised on nitrocellulose membrane were probed with relevant primary antibodies. Species specific secondary antibodies with an associated Horseradish peroxidase (HRP) activity were used to detect bound primary antibody. This interaction was visualised by enhanced chemiluminescence, ECL (Amersham). Immunoprobing was carried out as follows;

1. Membrane was washed for 5 minutes twice in wash buffer, PBS_A/0.1% Tween, with agitation on an orbital shaker (Stovall) which was used for all further washes.

2. Wash buffer was decanted and replaced with blocking buffer, 5% (w/v) Marvel (Premier Beverages) in wash buffer, and membranes were incubated for 1 hour at room temperature with constant agitation or overnight at 4°C.

3. Blocking buffer was removed and membranes were incubated with relevant primary antibody, diluted to the required working concentration in incubation buffer, 1% (w/v) Marvel in wash buffer, for ≥1 hour at room temperature.

4. Primary antibody solution was decanted and membranes were washed x3 with 5 minute incubations of wash buffer. Membranes were then incubated with suitable secondary antibody-HRP (Biorad) conjugate specifically raised against the isotype of the primary antibody. Depending on the species of primary antibody, secondary

antibodies were diluted 1 in 3,000-5,000 with incubation buffer and membranes were incubated for ≥1 hour at room temperature.

5. Following incubation with secondary antibody, membranes were washed x5 with 5 minute incubations of wash buffer. Antibody binding was visualised by ECL reagents. Following the final membrane wash, a 1:1 mix of ECL reagents 1 and 2 were overlayed on the membranes for 1 minute.

6. Membranes were blotted briefly to remove excess ECL reagent and exposed to autoradiographic Hyperfilm ECL (Amersham) for variable exposure times. Hyperfilm was developed in an automatic developer system (AGFA Curix 60 developer or an X-ograph Imaging Systems Compact x4 developer).

Gel Composition → Gel Components ↓	STACK (4%)	RESOLVING (7.5%)	RESOLVING (10%)
Bis acrylamide 30% w/v (37.5:1)	1.3ml	5.0ml	6.67ml
d.i. water	6.1ml	9.7ml	8.0ml
Tris.HCL 1.5M pH8.8	_	5.0ml	5.0ml
Tris.HCL 0.5M pH6.8	2.5ml	_	_
10% SDS	100µl	200µl	200µl
10% APS	50µl	100µl	100µl
TEMED	10µl	20µl	20µl

Table 3.3.1. Composition of SDS-PAGE stack and resolving gel

3.3.2. Immunoprecipitation of proteins from cultured cells

Immunoprecipitation was performed to purify proteins of interest and any associated proteins. Adherent cells were cultured as required prior to immunoprecipitation with either a conjugated or unconjugated protein specific antibody. Immunoprecipitation was performed as follows;

1. Adherent cells were dissociated from the tissue culture vessel with Accutase. Protease activity was inhibited by addition of growth medium containing 10% FCS and cell suspension was transferred to a 25ml universal tube and centrifuged at 1,500rpm for 8 minutes.

2. The supernatant was decanted and the cell pellet was resuspended in either 0.5 or 1ml of lysis buffer (0.5ml per $1x10^6$ cells or T75 flask, 1ml per $1x10^7$ cells or T175 flask) and transferred to a 1.5ml eppendorf tube. The lysis solution was gently mixed on a rotator at 4°C for 20 minutes to lyse the cells.

3. The lysate was centrifuged at 14,000rpm, 4° C for 20 minutes, supernatant was immediately transferred to fresh centrifuge tube, and the pellet was discarded. Cell lysate was pre-cleared with 50µl of a 50% slurry of agarose conjugated protein-A and incubated on a rotator at 4°C for 45 minutes.

4. Agarose conjugated protein-A was removed by centrifugation at 14,000rpm, 4°C for 5 minutes, supernatant was decanted to fresh centrifuge tubes and the pellet was discarded.

5. A relevant volume (1-20µl ml^{-1}) of immunoprecipitating antibody or 20-40µl ml^{-1} of a 50% slurry of immunoprecipitating antibody directly conjugated to protein-A-agarose was added to cell lysate and incubated on a rotator at 4°C for ≥ 2 hours.

6. Unconjugated immunoprecipitating antibody was captured by addition of 40µl of a 50% slurry of protein A agarose and incubation on a rotator at 4°C for 1 hour.

7. Immunocomplex bound to protein-A agarose was then collected by pulse centrifugation at 14,000rpm for 5 seconds. The supernatant was then discarded and the immunocomplex was washed three times with 800µl of ice-cold lysis buffer.

8. The pelleted beads were resuspended in 60µl of 2x SDS sample buffer and heated to 100°C for 5 minutes to dissociate immunocomplexes from the beads. Samples were centrifuged at 14,000rpm for 5 minutes to pellet beads.

3.3.3. Time constrained immunoprecipitation of proteins from cultured cells

The short length of time for some growth factor incubations required a rapid lysis step and therefore a modified protocol was used. Rapid immunoprecipitation was carried out as follows;

Adherent cells were washed once with ice-cold PBS_A and excess PBS_A was removed by aspiration. Ice-cold lysis buffer was added to the culture vessel, (1ml per $1x10^7$ cells or T175 flask, 0.5ml per $1x10^6$ cells or T75 flask). Adherent cells were scraped from the dish with a plastic cell scraper and lysis solution was then transferred to a centrifuge tube.

Immunoprecipitation was then carried out as for standard immunoprecipitation protocol.

3.3.4. Immunofluorescent staining of cultured cells

Adherent cell lines were cultured on 13mm round coverslips placed in 6 well plates at a seeding density of $2x10^5$ per well. To improve adherence and cell morphology,

HEK 293 cells were cultured on 13mm round coverslips coated with fibronectin (Sigma). Cells grown to the required density were treated as required before being fixed, permeabilised and stained as follows;

1. Culture medium was aspirated from the 6-well plate and coverslips were washed once with PBS_A. The wash solution was quickly aspirated and cells were fixed in 3.7% formaldehyde/PBS_A at room temperature for 20 minutes.

2. The fixing solution was then aspirated off and the coverslips were washed twice with PBS_A. Cells were then permeabilised with 0.2%Triton X-100/PBS_A at room temperature for 15 minutes.

3. Coverslips were then washed three times with PBS_A prior to incubation in blocking buffer 2% BSA/PBS_A for 20 minutes. 100µl of protein specific primary antibody diluted in blocking buffer, was overlayed onto each 13mm round coverslip and incubated at room temperature for 1 hour.

4. Coverslips were drained of antibody solution by dabbing onto tissue and then washed three times in PBS_A. 100µl of secondary antibody conjugated to a relevant fluorescent moiety diluted in blocking buffer was overlayed onto each 13mm round coverslip and incubated in the dark at room temperature for 1 hour.

5. Coverslips were drained of antibody solution by dabbing the coverslip edge onto tissue and then washed four times in PBS_A under low light conditions or darkness. Coverslips were mounted onto borosilicate glass slides with Fluor-guard and sealed with a dark non-fluorescent nail polish.

6. Specimens were examined on a Zeiss Axioplan upright microscope equipped for fluorescence using FITC and rhodamine filter sets. Cells were documented with a Nikon FX-35DX 35mm camera controlled by a UFX-DC camera controller. Alternatively, specimens were examined on a Zeiss Axiovert 135 inverted microscope using FITC and rhodamine filter sets and digitised using a Hammamatsu C1492 B/W CCD camera and image analysis software.

3.3.5. Staining of F-actin in cultured cells

Adherent cell lines were cultured as before on 13mm round coverslips placed in 6 well plates (Nuncleon) at a seeding density of $2x10^5$ per well. Cells grown to the required density were treated as required before being fixed, permeabilised and stained as follows;

1. Culture medium was aspirated from the 6-well plate and coverslips were washed once with PBS_A. The wash solution was quickly aspirated and cells were fixed in 3.7% formaldehyde/PBS_A at room temperature for 20 minutes.

2. The fixing solution was then aspirated off and the coverslips were washed twice with PBS_A. Cells were then permeabilised with 0.2%Triton X-100/PBS_A at room temperature for 15 minutes.

3. Coverslips were then washed three times with PBS_A. Phalloidin (Sigma) conjugated to either fluoroscein isothiocyanate (FITC) or tetramethyl-rhodamine isothiocyanate (TRITC) at a dilution of 1/500 in PBS_A was overlayed onto each 13mm round coverslip and incubated in the dark at room temperature for 45 minutes.

4. Coverslips were drained of phalloidin-conjugate by dabbing the edge of the coverslip onto tissue and then washed four times in PBS_A under low light conditions or darkness.

5. Coverslips were mounted onto borosilicate glass slides using either citifluor or Fluor-guard and sealed with a dark non-fluorescent nail polish. Specimens were examined on a Zeiss Axioplan or Axiovert microscope equipped for fluorescence.

3.3.6. Fluorescence activated cell sorting (FACS) analysis of cultured cells

1. Adherent cells were dissociated from culture vessels and washed with 10ml of PBS_A. Cell suspensions were centrifuged on a Beckman GS-6 bench-top centrifuge at 1,400rpm for 6 minutes.

2. The supernatant was discarded and cells were resuspended at a cell density of $2x10^6$/100µl PBS_A. 300µl of cell suspension was aliquoted to 5ml FACS tubes. Cells were incubated with relevant protein specific antibody, on ice for 1 hour. Cells were washed three times with PBS_A and re-suspended in 300µl PBS_A.

3. Cells were incubated with a 1/50-100 dilution of fluorophore-labelled secondary-antibody specific for the primary antibody isotype at 4°C for 1 hour. Cells were washed three times with PBS_A and resuspended in 250µl PBS_A ready for analysis by FACS.

3.3.7. Immunofluorescent assay for the incorporation of BrdU into DNA

Measurement of cell proliferation was performed by a non-radioactive, fluorescence based method first proposed by (Gratzner, 1982). 5'-bromo-2'-deoxy-uridine can be incorporated into DNA in place of thymidine during *de novo* DNA synthesis. Incorporated BrdU can then be detected with a monoclonal antibody developed against BrdU and a fluorochrome-conjugated second antibody (BrdU Detection Kit I, Boehringer Mannheim).

1. Adherent cells were cultured on 13mm round coverslips in 6-well tissue culture dishes until 50% confluent. Cells were then treated as required.

2. Cell culture medium was removed by aspiration and replaced with 2 ml of culture medium containing 0.1% (v/v) BrdU labelling reagent (I). Cells were incubated at 37°C, 5% CO_2 for 1 hour.

3. Culture medium was removed by aspiration and cells were washed three times in washing buffer (V). Cells were fixed in ice cold 70% (v/v) ethanol/50mM Glycine buffer (pH2.0) at -20°C for 30 minutes.

4. Cells were washed three times in washing buffer (V) and were then covered with anti-BrdU working solution (II) and incubated at 37°C for 30 minutes.

5. Cells were washed three times in washing buffer (V) and were then covered with anti-mouse-Ig-fluorescein working solution (IV) and incubated at 37°C for 30 minutes.

6. Cells were washed three times in washing buffer (V). Cover-slips were mounted onto glass slides with Fluoro-guard and sealed with nail polish. Cells were examined using a Zeiss Axioplan microscope using the FITC filter set and documented with a Nikon FX-35DX 35mm camera controlled by a UFX-DC camera controller.

3.3.8. Immunofluorescent analysis of apoptotic cells

One of the early events in most cell types during apoptosis is the translocation of phosphatidylserine from the inner surface of the plasma membrane to the cell surface (Martin et al., 1995). Phosphatidylserine can then be easily detected in living cells with a FITC conjugate of annexin V. The ApoAlert ™ Annexin V Apoptosis Kit from Clontech was used for apoptosis detection.

1. Cells were cultured on either 13mm round coverslips or in 8-well chamber slides (Nuncleon) and treated as desired.

2. Cells were washed once with PBS_A and coverslips were overlayed with 100µl of 1x binding buffer containing 2.5% (v/v) enhanced annexin V-FITC (0.5µg ml^{-1} final) and 2.5% (v/v) propidium iodide.

3. Cells were incubated in the dark at room temperature for 15 minutes. Cells were washed twice with PBS_A and fixed with 2% (v/v) formaldehyde/PBS_A for 15 minutes.

4. Cells were washed twice with PBS_A and mounted in Fluoro-guard. Fluorescence was observed with a Zeiss Axioplan microscope using FITC and rhodamine filter sets. Fluorescent images were documented with a Nikon FX-35DX 35mm camera controlled by a UFX-DC camera controller.

3.3.9. Measurement of PI 3 kinase activity

The activation of PI 3-kinase enzymatic activity is required for various signalling pathways in macrophages. PI 3-kinase activity can be isolated from cells by immunoprecipitation of the p85/p110 complex with antibodies raised against p85α. PI 3-kinase activity was measured in vitro by incorporation of ^{32}P-labelled γATP (Sigma) onto the PI 3-kinase substrates PI and PI_4P.

1. Bac1.2F5 cells were cultured to 80% confluency in T75 tissue culture flasks and quiesced for 24 hours by depriving the cells of M-CSF. Cells were incubated as required, then culture medium was quickly removed by aspiration and the culture flasks were placed on ice.

2. 750μl of Lysis buffer was added to each T75 flask, cells were detached by scraping, and the lysate was transferred to a 1.5ml microcentrifuge tube. Samples were incubated on a rotator at 4°C for 30 minutes.

3. Lysate was centrifuged at 14,000rpm, 4°C for 30 minutes to sediment insoluble material and the supernatant was decanted to a fresh microcentrifuge tube. Samples were incubated with 50μl of a 50% slurry of anti-p85α-protein-A-agarose conjugate (UBI) at 4°C on a rotator for 1 hour.

4. The immunoprecipitate was collected by pulse centrifugation at 14,000rpm and supernatant was decanted. The immunoprecipitate was washed three times with lysis buffer, once with 50mM HEPES (pH7.5) and once with (X1) PI 3-kinase assay buffer.

7. Immunoprecipitate was re-suspended in 25µl of (X2) PI 3-kinase buffer and 10µl of a PI/PI_4P mixed lipid micelle suspension (1mg ml^{-1}) were added. 25µl of assay mix were added and the samples were incubated at 37°C for 10 minutes.

8. The reaction was quenched by adding 100µl of 6M HCl. 200µl of a chloroform:methanol (1:1) solution was added, vortexed for 20 seconds and centrifuged at 14,000rpm for 2 minutes. The upper aqueous phase was discarded and 80µl of 1M HCl:methanol (1:1) was added, vortexed for 20 seconds and centrifuged at 14,000rpm for 2 minutes.

9. The upper aqueous phase was discarded again and the lower organic phase was dried down by centrifugation in a speed-vac for 4 minutes.

10. Samples were re-suspended in 20µl of chloroform and spotted onto a 60Å silica gel chromatography plate. Lipid standards were also spotted onto the plate for comparison. Plates were developed by chromatography in a solvent system containing 50% (v/v) chloroform, 39% (v/v) methanol and 11% (v/v) 4M ammonia solution.

11. Chromatography plates were air dried and lipids and lipid markers were visualised by exposing the plates to iodine crystals for 2-3 minutes. Radiolabelled lipids were detected either by autoradiography or by real-time detection on a Phosphoimager (Canberra Packard).

Plate preparation:

Thin layer chromatography plates (TLC) were activated by pre-running in a solvent system containing 40% (v/v) methanol, 1mM EDTA, 1% potassium oxalate in d.i. water. Plates were then heated to 110°C for 20 minutes immediately before use.

Lipid preparation:

Phosphatidylinositol lipids were obtained either in chloroform solution or as lyophilised powder. Lipids were dried down under a stream of nitrogen and re-suspended in chloroform to 1mg ml^{-1}. The required amount of lipid was added to a fresh microcentrifuge tube and the lipids were dried down under a stream of nitrogen before being re-suspended in (X1) PI 3-kinase buffer. Lipids were re-suspended, ready for use, by sonication in an ice bath for 5 minutes.

Serum Delipidation:

Serum was delipidated by mixing equal volumes of foetal bovine serum (FBS) with chloroform/methanol (2:1 v/v) for 2 hours at room temperature. The organic and aqueous phases were separated by centrifugation on a Beckman J2-MC at 3,000rpm for 5 minutes. The delipidated aqueous was removed and residual organic solvents were removed by applying a water-aspirator vacuum for 2 hours. The resultant delipidated FBS was filter sterilised for later use.

3.4.0 Molecular biology

3.4.1. Isolation of total RNA

Total RNA was purified from mammalian cells to be used as a template for reverse transcription-PCR of cDNA with gene specific oligonucleotide primers.

1. Cultured cells were washed twice with PBS_A and $1x10^6$ cells were lysed in 100µl RNA buffer D. 100µl of 2M sodium acetate (pH4.0) was added to the lysate and mixed by inversion. An equal volume of equilibrated phenol was added and the solution was mixed by inversion until clear.

2. 200µl of chloroform per ml of lysate was added, mixed by vigorous shaking for 10 seconds and incubated at 4°C for 10 minutes. Lysate was centrifuged at 14,000rpm, 4°C for 20 minutes. The aqueous phase was decanted to a fresh microcentrifuge tube and an equal volume of propan-2-ol was added and mixed. Samples were chilled to -20°C for ≥ 1 hour and centrifuged at 14,000rpm, 4°C for 20 minutes.

3. The RNA pellet was re-suspended in 300µl per ml of original lysate volume. An equal volume of propan-2-ol was added and the samples were chilled to -20°C for ≥ 1 hour.

4. Samples were centrifuged at 14,000rpm, 4°C for 10 minutes and the supernatant was decanted. The pellet was washed with 300µl 75% ethanol and centrifuged at 14,000rpm, 4°C for 10 minutes. The supernatant was decanted and the pellet was dried in a speed-vac for 4 minutes.

5. The RNA pellet was re-suspended, by pipetting, in 50µl DEPC-treated d.i. water by heating to 65°C to aid solubilisation.

3.4.2. Amplification of DNA by PCR

Amplification of specific DNA fragments was carried out by polymerase chain reaction (Mullis and Faloona, 1987) as follows;

PCR amplification of DNA with AmpliTaq DNA polymerase:

Each PCR reaction was set up as follows;

	Volume	Final Concentration
d.i. water to a final volume of	100µl	
10X PCR buffer	10µl	1X
dNTPs (10mM)	8µl	200µM
AmpliTaq DNA polymerase	0.5µl	2.5 Units/100µl
5′→3′ Primer (Forward)	Xµl	1.0µM
3′→5′ Primer (Reverse)	Xµl	1.0µM
Template	Xµl	1ng/100µl

Reactions were pulse centrifuged and overlayed with mineral oil.

Initially the volume of each primer required was calculated to obtain a final concentration of 1.0µM. However, the final concentration of primers and template DNA were further optimised to improve the quality of amplified products. Negative controls were performed for each reaction and a known positive control was included to demonstrate that the PCR system was functioning properly. PCR was performed on a Techne PHC-3 air-cooled thermocycler under varying cycling parameters. A typical reaction set up was as follows;

	Temperature	Time	No. Cycles
Denaturation	94°C	2 min	x1
Denaturation	94°C	1 min	
Annealing	55°C	1 min	x32
Extension	72°C	30 sec	
Extension	72°C	7 min	x1

Reverse Transcription PCR amplification with AmpliTaq DNA polymerase:

Each Reverse transcription reaction was set up as follows;

	Volume	Final Concentration
MgCl solution (25mM)	4µl	5mM
10X PCR buffer II	2µl	1X
dNTPs	8µl	1mM
Rnase inhibitor	1µl	1 U/µl
Oligo $d(T)_{16}$	1µl	2.5 U/µl
RNA template (U937 total RNA)	1µl	1.0µg/20µl
DEPC treated d.i. water to	20µl	

Reactions were pulse centrifuged and overlayed with mineral oil.

Reverse transcription was performed on a Techne PHC-3 for one cycle as follows;

	Temperature	Time
Pre-incubation	25°C	10 min
Reverse Transcribe	42°C	15 min
Denature	99°C	5 min
Cool	5°C	5 min

Each PCR reaction mix was set up as follows and then added to the RT reaction;

	Volume	Final Concentration
MgCl solution (25mM)	4µl	2mM
10X PCR buffer II	8µl	1X
d.i. water	65.5µl	
AmpliTaq DNA polymerase	0.5µl	2.5 U/100µl
5′→3′ Primer (Forward)	1µl	0.15µM
3′→5′ Primer (Reverse)	1µl	0.15µM
Total volume:	80µl	

Reactions were pulse centrifuged and PCR amplification was performed on a Techne PHC-3 under the following conditions;

	Temperature	Time	No. Cycles
Denaturation	94°C	2 min	x1
Denaturation	94°C	1 min	
Annealing	55°C	1 min	x35
Extension	72°C	30 sec	
Extension	72°C	7 min	x1

Colony PCR for identification of positive clones:

Each PCR reaction was set up as follows;

	Volume	Final Concentration
d.i. water to a final volume of	25µl	
10X PCR buffer	2.5µl	1X
dNTPs (10mM)	2µl	200µM
AmpliTaq DNA polymerase	0.125µl	2.5 Units/100µl
5′→3′ Primer (Forward)	Xµl	1.0µM
3′→5′ Primer (Reverse)	Xµl	1.0µM
Template:	A single colony or 1µl of inoculated LB broth.	

Reactions were pulse centrifuged and overlayed with mineral oil.

PCR was performed on a Techne PHC-3 air-cooled thermocycler under varying cycling parameters. A typical reaction set up was as follows;

	Temperature	Time	No. Cycles
Denaturation	94°C	2 min	x1
Denaturation	94°C	1 min	
Annealing	55°C	1 min	x25
Extension	72°C	30 sec	
Extension	72°C	7 min	x1

PCR amplification of DNA with Advantage™ KlenTaq polymerase mix:

The simultaneous use of two different DNA polymerases, a primary and proof-reading enzyme, in a PCR reaction was employed to amplify significantly longer fragments, with higher fidelity and increased yield. Each PCR reaction was set up as follows;

	Volume	Final Concentration
d.i. water to a final volume of	50µl	
10X KlenTaq PCR reaction buffer	5µl	1X
dNTPs (10mM)	2µl	40µM
50X Advantage KlenTaq mix	1µl	1X
5′→3′ Primer (Forward)	Xµl	0.4µM
3′→5′ Primer (Reverse)	Xµl	0.4µM
Template	Xµl	1ng/50µl

Reactions were pulse centrifuged and overlayed with mineral oil.

Negative controls were performed for each reaction and a known positive control was included to demonstrate that the PCR system was functioning properly. PCR was performed on a Hybaid Omnigene air-cooled thermocycler under varying cycling parameters. A typical reaction set up was as follows;

	Temperature	Time	No. Cycles
Denaturation	94°C	1 min	x1
Denaturation	94°C	30 sec	
Annealing/Extension	68°C	6 min	x25
Extension	68°C	5 min	x1

3.4.3. Separation of DNA fragments by agarose gel electrophoresis

Agarose gels for the resolving of DNA fragments were made by dissolving 0.7-1.2% w/v electrophoresis grade agarose (Gibco-BRL) in TBE buffer and microwaved to solubilise the agarose. Agarose gels were cast in either a BRL Horizon 11.14 mini-gel casting tray or a BRL Horizon 58 midi-gel tank. After gels had polymerised they were placed in TBE buffer. DNA samples were mixed with 10x DNA loading buffer and applied to the gel. The DNA gel was run at constant voltage for the desired length of time. DNA fragments were visualised under ultra-violet light by including 0.5ng/µl Ethidium Bromide in the agarose gel during casting. Alternatively, DNA was stained following electrophoresis by submerging the gel in TBE buffer containing SYBER™ Green I (1:10,000 dilution) for 30 minutes. DNA agarose gels were visualised on a UVP transilluminator and documented with a Polaroid™ land camera. Molecular weight markers (1kBp ladder, Gibco-BRL) were included on all gels.

3.4.4. Purification of DNA from agarose gels

A clean source of recombinant DNA was required for downstream applications such as PCR, DNA ligation, bacterial transformation and sequencing. DNA was resolved by agarose gel electrophoresis and DNA bands of the correct molecular weight was excised from the gel and purified by the QIAquick method (QIAGEN) as follows;

1. Each DNA fragment was excised from the agarose gel with a sterile scalpel and placed in a clean 1.5ml microcentrifuge tube and the gel slice weight was determined.

2. Three volumes of buffer QX1 was added to one volume of gel, (100mg ≈ 100µl). Samples were incubated at 50°C for 10 minutes and were mixed by inverting the tube to dissolve the gel slice. One volume of room temperature isopropanol was added and mixed by inverting the tube.

3. A QIAquick spin column was placed in a 2ml collection tube and the sample was transferred from the microcentrifuge tube to the spin column and centrifuged at 14,000rpm for 1 minute. Flow-through was discarded and the spin column was placed back in the same collection tube.

4. 0.5ml of buffer QX1 was added to the spin column and the sample was centrifuged at 14,000rpm for 1 minute. Flow-through was discarded and the spin column was placed back in the same collection tube.

5. The spin column was washed with 0.75ml of buffer PE and the sample was centrifuged at 14,000rpm for 1 minute. Flow-through was discarded and the spin

column was placed back in the same collection tube and recentrifuged at 14,000rpm for 1 minute.

6. The QIAquick column was placed in a clean 1.5ml microcentrifuge tube and DNA was eluted from the column by applying 30µl of d.i. water to the middle of the spin column. The column was allowed to stand for 1 minute and then centrifuged at 14,000rpm for 1 minute to collect eluted DNA.

3.4.5. Restriction enzyme digestion of DNA

Restriction enzyme digests were carried out on plasmid DNA for either diagnostic or sub-cloning purposes. Restriction enzymes were supplied with their relevant x10 buffers and all digests were performed on 2-3µl of DNA obtained from either mini-preps or midi-preps at 37°C for ≥ 1 hour. In all reactions the glycerol concentration never exceeded 5% (v/v).

3.4.6. Ligation of PCR products into pCR2.1

DNA fragments of the expected size obtained from PCR reactions were gel purified prior to ligation into pCR2.1 cloning vector. Ligation ratios of PCR product to plasmid vector of 1:1 and 1:3 were used and ligation of PCR products into pCR2.1 was performed in 0.5ml microcentrifuge tubes at 14°C for ≥ 4 hours. Ligation reactions were set up as follows;

	Volume
Fresh PCR product	X µl
10X Ligation buffer	1 µl
pCR2.1 vector (25ng/µl)	2 µl
T4 DNA ligase (4.0 Weiss units)	1 µl
d.i. water to	10 µl

3.4.7. Transformation of Competent E. coli

Plasmid DNA containing clones of interest were transformed into the relevant E. coli strain. Maintenance of bacterial stocks was required for preparation of plasmid DNA for downstream applications such as sub-cloning, sequencing and transfection. Bacteria were transformed as follows;

1. Bacteria were thawed on ice and either 50 or 100µl of competent cells were aliquoted into polypropylene microcentrifuge tubes.

2. 2µl of β-mercaptoethanol was gently pipetted into each vial of competent cells and mixed by gentle stirring. 2µl of each ligation reaction was pipetted into each vial of competent cells and mixed by gentle stirring. Vials were incubated on ice for 30 minutes.

3. Each vial containing competent cells was heat shocked at 42°C for exactly 30 seconds and immediately placed on ice for a further 2 minutes. 250µl of room temperature SOC medium was added to each vial and incubated on a rotary shaking incubator at 225rpm, 37°C for 1 hour.

4. 50µl and 200µl from each transformation vial were plated out on LB agar plates containing 50µg ml^{-1} carbenicillin. Plates were allowed to absorb the transformation liquid before inverting them and were incubated at 37°C for 18 hours.

3.4.8. Selection of positive clones by alpha-complementation

In pCR2.1 vector there are short segments of E. coli DNA containing regulatory sequences and coding information for the amino-terminal 146 a.a. of the β-galactosidase gene (*lac* Z). Host cell strains, which possess coding regions of the carboxyl-terminal portion of the β-galactosidase gene, can then be transformed with pCR2.1. Both the host cell and vector portions of the β-galactosidase gene can associate to form a polypeptide that is enzymatically active. Bacteria that have been transformed with wild type vector are recognised by formation of blue colonies in the presence of chromogenic substrate, X-gal. However, ligation of PCR product into the multiple cloning site of pCR2.1 disrupts production of the amino-terminal fragment of β-galactosidase and therefore bacteria containing recombinant plasmids are identified as white colonies. Two E. coli strains were employed as host cells for cloning purposes, INVαF′ and TOP10F′. The E. coli strain INVαF′ required LB plates spread with 40μl of a 40mg ml^{-1} stock of X-gal and TOP10F′ required′ required LB plates spread with 40μl each of 40mg ml^{-1} X-gal and 100mM IPTG.

3.4.9. Small scale purification of plasmid DNA

Small scale plasmid purification was performed using Qiaprep spin miniprep kit (Qiagen), a modification of the method introduced by Birnboim & Doly (1979).

1. Positive single colonies (white) were selected and inoculated into 5ml of LB broth containing 50μg ml^{-1} Carbenicillin (Sigma) and incubated at 37°C with shaking for 16-18 hours.

2. 1.5-2.0ml of bacterial culture was centrifuged at 14,000rpm for 2 minutes in sterile 2ml eppendorf tubes. Supernatants were removed by aspiration. Bacterial pellets were resuspended completely in 250µl of buffer P1. 250µl of buffer P2 was added and tubes were mixed by inverting 5-6 times.

3. The lysis reaction was then stopped by addition of 350µl of buffer N3 and was mixed by inverting 5-6 times. The suspension was then centrifuged at 14,000rpm for 10 minutes to pellet the precipitate.

4. The supernatant was decanted to a Qiaprep spin column seated in a 2ml collection tube. The samples were centrifuged at 14,000rpm for 1 minute. Flow-through was discarded and 0.5ml of buffer PB was added to the Qiaprep spin columns and centrifuged at 14,000rpm for 1 minute.

5. Flow-through was discarded and 0.75ml of buffer PE was added to the Qiaprep spin columns and centrifuged at 14,000rpm for 1 minute. Columns were then centrifuged for an additional 1 minute to remove residual wash buffer.

6. The Qiaprep spin column was then placed in a clean 1.5ml eppendorf tube. DNA was eluted by adding 30µl of d.i. water to the centre of the Qiaprep column, leaving to stand for 1 minute before centrifugation at 14,000rpm for 1 minute.

3.4.10. Medium scale purification of plasmid DNA

The HYBAID RECOVERY™ Plasmid Midi Prep Kit was employed for isolation of up to 200µg of plasmid DNA from high copy plasmid vectors.

1. 10-30ml of bacterial culture was prepared and the bacteria suspension was pelleted by centrifugation at 3,200rpm on a table top centrifuge for 12 minutes. Supernatant was decanted and the bacterial pellet was resuspended in 500µl d.i. water and transferred to a 2ml microcentrifuge tube.

2. The bacteria were pelleted by centrifugation at 14,000rpm for 30-60 seconds and supernatant was decanted. Pellets were further pulse centrifuged and any remaining liquid was removed. 200µl of pre-lysis solution was added and mixed by vortexing until the cells were completely resuspended.

3. 400µl of alkaline lysis solution was added and the tubes were gently inverted 15 times. Tubes were incubated at room temperature for 3 minutes. 300µl of ice-cold neutralising solution was added and vortexed until a uniform white precipitate was observed. The samples were then placed on ice for 5 minutes.

4. Precipitate was pelleted by centrifugation at 14,000rpm for 5 minutes. The supernatant was transferred to a clean 2ml microcentrifuge tube. The silica gel matrix in the binding buffer was resuspended by vigorous shaking and 1.2 ml was immediately added to the bacterial lysate.

5. The tubes were gently inverted 15 times to ensure uniform mixing. Samples were then incubated at room temperature for 5 minutes with further mixing on a rotator.

The binding matrix/DNA complex was pelleted by a brief pulse spin and the supernatant was discarded.

6. 500µl of wash solution was added to the binding matrix/DNA complex and resuspended by gentle stirring with a pipette tip and by pipetting up and down. The suspension was then transferred to a spin filter and centrifuged at 14,000rpm for 30 seconds to transfer wash solution into the catch tube.

7. The catch tube was emptied and another 500µl wash solution was added to the spin filter and pulsed for an additional 20 seconds. The catch tube was emptied and the filter was centrifuged for 2-5 minutes to dry the filter contents.

8. The spin filter was then inserted into a new catch tube and 300µl of d.i. water was added to the spin filter and the binding matrix/DNA complex was resuspended by gentle stirring with 200µl a pipette tip. The spin filter was centrifuged at 14,000rpm for 5-8 minutes and eluted DNA was collected in the catch tube.

3.4.11. Large scale purification of plasmid DNA

Large scale plasmid preps for preparations of up to 500µg of DNA were performed using the QIAGEN Endofree maxi-prep column, with the following protocol;

QIAGEN™ EndoFree Plasmid Maxi protocol:

1. 100ml of bacterial culture were centrifuged in a JC-10 rotor, on a Beckman J2-MC centrifuge, at 5,000rpm, 4°C for 15 minutes. The bacterial pellet was resuspended completely in 10ml of buffer P1.

2. 10ml of buffer P2 was added and was mixed gently by inverting 5-6 times then left at room temperature for 5 minutes. 10ml of chilled buffer P3 was added to the lysate and was mixed immediately but gently by inverting 5-6 times. The lysate was poured into the barrel of the QIAfilter cartridge that had been capped at the outlet nozzle and incubated at room temperature for 10 minutes.

3. The cap on the outlet nozzle was removed to allow flow-through, the plunger was gently inserted, and the lysate was filtered into a 50ml Falcon tube. 2.5ml of buffer ER was added to the filtered lysate and mixed by inverting the tube 10 times. Lysate was then incubated on ice for 30 minutes.

4. A QIAGEN-tip 500 was equilibrated by applying 10ml of buffer QBT and allowed to empty by gravity flow. The cleared lysate from step 5 was then applied to the QIAGEN-tip and allowed to enter the resin by gravity flow. The QIAGEN-tip was then washed with two 30ml volumes of buffer QC.

5. Plasmid DNA was eluted from the QIAGEN-tip into a 50ml Falcon tube with 15ml of buffer QN. DNA was precipitated by addition, with mixing, of 10.5ml of room temperature isopropanol. Solution was centrifuged immediately in a JC-20 rotor at 11,000rpm, 4°C for 30 minutes.

6. The supernatant was carefully decanted and the DNA pellet was washed with 2.5ml of endotoxin free, room temperature 70% ethanol and recentrifuged at 11,000rpm, 4°C for 10 minutes.

7. The supernatant was carefully decanted so not to disturb the DNA pellet and then the pellet was air dried for 5-10 minutes. The plasmid DNA pellet was redissolved in 500µl of endotoxin free, d.i. water and transferred to a clean microcentrifuge tube.

3.4.12. Hydrolysis of 5′-phosphate groups from DNA

During the ligation of cloned DNA into a mammalian expression vector, removal of the 5′-phosphate group from linearised vector DNA was required to prevent recircularisation and religation in the ligation reaction. The 5′-phosphate group on the 5′-protruding end of vector DNA was removed with calf intestinal alkaline phosphatase (CIAP) enzyme.

1. DNA to be dephosphorylated was set up as follows in a 0.5ml microcentrifuge tube;

DNA (up to 10pmol of 5′ ends)	40µl
CIAP 10X reaction buffer	5µl
CIAP (0.01 U/µl)	5µl

2. Samples were incubated at 37°C for 30 minutes. Another 5µl of CIAP (0.01 U/µl) was added and the samples were incubated for a further 30 minutes at 37°C.

3. The reaction was terminated by the addition of 300µl of CIAP stop buffer and heat inactivated at 75°C for 10 minutes prior to gel purification of the DNA fragment.

3.4.13. Identification of positive clones by colony-PCR

Identification of positive clones by colony-PCR was performed when either a large population of clones were to be screened or when colour selection by alpha-complementation was not suitable. Oligonucleotides specific for either vector

sequence or internal, gene specific oligonucleotides were used to identify positive clones as follows;

1. Colonies were picked with an inoculation loop and streaked to a master plate before being inoculated directly into the PCR reaction. PCR reactions were set up as follows;

	Volume	Final Concentration
10X PCR buffer	5µl	1X
dNTPs (10mM)	4µl	200µM
5′→3′ Primer (Forward)	Xµl	1.0µM
3′→5′ Primer (Reverse)	Xµl	1.0µM
AmpliTaq DNA polymerase	0.25µl	1.25 Units/50µl
d.i. water to a final volume of		50µl

2. PCR reactions were overlayed with mineral oil and cycled as follows;

	Temperature	Time	No. Cycles
Denaturation	94°C	2 min	x1
Denaturation	94°C	1 min	
Annealing	55°C	1 min	x25
Extension	72°C	1 min	
Extension	72°C	7 min	x1

3. PCR products were analysed by DNA agarose-gel electrophoresis.

3.4.14. Site-directed mutagenesis of specific nucleotides

Point mutations were introduced into a cloned gene using the QuikChange™ site-directed mutagenesis (SDM) kit (Stratagene). The QuikChange SDM method was performed using *Pfu* DNA polymerase and two complimentary oligonucleotide primers containing the desired mutation flanked by unmodified sequence, which had been HPLC purified. Site-directed mutagenesis for each mutation site was performed as follows;

1. Sample and control reactions were set up in 0.5ml microcentrifuge tubes as follows;

10X reaction buffer	5µl
dsDNA template (20ng)	Xµl
5′→3′ oligonucleotide primer 1 (125ng)	Xµl
3′→5′ oligonucleotide primer 2 (125ng)	Xµl
dNTP mix	1µl
d.i. water to a final volume of	50µl

2. 1µl of *Pfu* DNA polymerase (2.5 U/µl) was added, stirred gently to mix and overlayed with mineral oil. Reaction tubes were placed on a Hybaid thermal cycler and cycling parameters were set up as follows;

	Temperature	Time	No. Cycles
Segment 1	95°C	30 sec	x1
	95°C	30 sec	
Segment 2	55°C	1 min	x12
	68°C	17 min	

3. After temperature-cycling, reactions were placed on ice for 2 minutes and then 1µl of the restriction enzyme *Dpn* I (10 U/µl) was pipetted into each reaction tube, below the mineral oil overlay, and mixed by pipetting up and down several times.

4. The reaction tubes were centrifuged at 14,000rpm for 1 minute and incubated at 37°C for 1 hour to digest parental supercoiled dsDNA.

5. 1µl of *Dpn* I treated DNA was transformed into 50µl of E. coli XL1-Blue supercompetent cells. The transformation was performed as in section 3.4.6. however, cells were heat shocked for 45 seconds at 42°C.

6. All of the transformation reaction was plated onto LB agar plates containing 50µg ml^{-1} carbenicillin and the plates were allowed to absorb the transformation before

incubation at 37°C for 18 hours. Colonies were selected and inoculated into LB-carbenicillin broth. Plasmid mini-preps were prepared and inserts were sequenced to confirm clones containing required mutation.

3.4.15. Nucleotide sequence analysis

The DNA sequences of cloned DNA were determined following the ABI Prism™ dRhodamine terminator cycle sequencing ready reaction kit on either an ABI Prism 310 Genetic Analyzer or an ABI Prism 377 DNA sequencer (Oswel).

DNA purification:

1. DNA prepared from a QIAGEN mini-prep column was made up to 50µl with d.i. water and mixed with 30µl of 20% (v/v) polyethylene glycol (PEG)/2.5M NaCl and incubated at 4°C for 30 minutes.

2. Samples were centrifuged at 14,000rpm at 4°C for 15 minutes. The supernatant was decanted and the pellet was washed gently with 70% (v/v) ethanol/H_2O. The 70% ethanol/H_20 was decanted and the pellet was dried in a vacuum centrifuge for 2 minutes.

3. The DNA pellet was resuspended in 20µl of d.i. water and DNA concentration was calculated from an OD_{260nm} absorbance reading taken on an Beckman DU-64 spectro-photometer.

Thermal cycling with dRhodamine terminators:

1. For each reaction the following reagents were added to a 0.5ml thin-walled PCR tube,

Terminator ready reaction mix	8µl
dsDNA template (0.2µg/µl)	1µl
Sequencing primer (3.2pmol/µl)	1µl
d.i. water	10µl

2. Reactions were mixed by pipetting and pulse centrifuged then overlayed with mineral oil. Samples were thermal cycled on a Hybaid and cycling parameters were as follows;

Temperature	Time	No. Cycles
96°C	30 sec	
50°C	15 sec	x25
60°C	4 mins	

4. Samples were then placed on ice prior to purification of extension products.

Purification of extension products:

1. The 20µl PCR reaction was removed to a clean 0.5ml microcentrifuge tube containing 10µl of 3M sodium acetate (pH4.6) and 250µl of 95% (v/v) ethanol/H_2O. Samples were vortexed briefly and incubated on ice for 10 minutes.

2. Samples were centrifuged at 14,000rpm, 4°C for 30 minutes. The supernatant was carefully removed by aspiration and the DNA pellet was washed gently with 250µl 70% (v/v) ethanol/H_2O, then centrifuged at 14,000rpm, 4°C for 30 minutes. Supernatant was carefully removed by aspiration and pellet was dried in a vacuum centrifuge for 2 minutes.

3. The DNA pellet was resuspended by repeated pipetting in 25µl of template suppression reagent for sequencing on the ABI 310 genetic analyzer or in 12µl of template suppression reagent for sequencing on the ABI 377 DNA sequencer.

4. Samples prepared for sequencing on the ABI Prism 310 were denatured by heating at 95°C for 2 minutes then were placed on ice until ready for loading. Samples for sequencing on the ABI Prism 377 were sent immediately for sequencing to Oswel DNA Service, Southampton, England.

3.4.16. *In vitro* translation

The procedure for synthesising proteins in vitro from cloned genes was first described by Hope and Struhl, 1985. In vitro translation involves transcription from the T7 promoter sequence in the plasmid vector containing the gene of interest. This yields mRNA that is subsequently translated using wheat germ extract or reticulocyte lysate. By using [^{35}S] methionine during the translation reaction, the protein is synthesised as a radiolabeled species and can be analysed by SDS-PAGE. The protocols used for synthesising [^{35}S] protein by in vitro transcription and translation are detailed below.

TNT® Coupled Wheat Germ Extract system (Promega)

1. TNT® RNA Polymerase was placed on ice and the TNT® Wheat Germ Extract was rapidly thawed by hand warming, then placed on ice. Other components were thawed at room temperature, then stored on ice.

2. Reaction components were set up in a 1.5ml microcentrifuge tube as below.

Component	Standard [^{35}S] methionine Reaction
TNT® Quick Master Mix	40µl
[^{35}S] methionine (1000Ci/mmol at 10mCi/ml)	2µl
DNA template (0.5µg/µl)	2µl
Nuclease-Free Water to a final volume of	50µl

3. Reactions were incubated at 30°C for 90 minutes then analysed in the same way as products from the TNT® Coupled Wheat Germ Extract system.

TnT® Coupled Wheat Germ Extract system (Promega)

1. TnT® RNA Polymerase was placed on ice and the TnT® Wheat Germ Extract was rapidly thawed by hand warming, then placed on ice. Other components were thawed at room temperature, then stored on ice.

2. Reaction components were set up in a 1.5ml microcentrifuge tube as below.

Component	Standard [^{35}S] methionine Reaction
TnT® Wheat Germ Extract	25µl
TnT® Reaction Buffer	2µl
TnT® RNA Polymerase (T7)	1µl
Amino Acid Mixture, Minus Leucine, 1mM	–
Amino Acid Mixture, Minus Methionine, 1mM	1µl
[^{35}S] methionine	2µl
RNasin® Ribonuclease Inhibitor, 40u/µl	1µl
DNA template	2µg
Nuclease-Free Water to a final volume of	50µl

3. The reaction was mixed gently after addition of each component and centrifuged briefly to collect the reaction at the bottom of the tube. Reactions were incubated at 30°C for 120 minutes.

4. Once the translation reaction was complete a 5µl aliquot was removed and added to 20µl of 2x SDS sample buffer. The remainder of the reaction was stored at -20°C.

5. Samples were heated to 100°C for 2 minutes to denature the proteins then resolved by SDS-PAGE.

6. The protein gel was soaked in 7% (v/v) acetic acid, 7% (v/v) methanol and 1% (v/v) glycerol for 5 minutes. The protein gel was then placed on a sheet of Whatman® 3MM filter paper, covered with plastic wrap and dried at 80°C for 30-90 minutes

under a vacuum using a conventional gel dryer. The preserved gel was exposed to autoradiographic film at -70°C for a minimum of 4 hours.

3.4.17. Transfection of mammalian cells

Various transfection methodologies for the introduction of DNA into mammalian cells were analysed to determine the most reproducible and efficient method of DNA transfer with the lowest toxicity to the cell-line being transfected. Various chemical and cationic lipid transfection products were investigated including, Calcium phosphate (ProFection™, Promega) CellFectin™, LipoFectin™, LipoFectamine™, DMRIE-C™ (Gibco-BRL) and SuperFect™ (QIAGEN). The protocols for Profection™, SuperFect™ and LipoFectAMINE™ are detailed below, all other protocols were carried out as per manufacturers protocols.

Calcium phosphate mediated transfection:

Transfection of recombinant DNA mediated by calcium phosphate was performed with the ProFection™ kit based on the observation originally described by Graham and van der Eb, 1973.

1. Cultured cells were seeded at $2x10^5$/well in a 6-well culture plate. Cells were left to adhere for 24-36 hours.

2. Three hours prior to transfection the culture medium was removed and replaced with 3ml of fresh growth medium. Transfection reagents were thawed, equilibrated to room temperature and mixed thoroughly. For each transfection two 1.5ml microcentrifuge tubes were set up as follows;

		per 6-well
Tube 1	DNA (1μg/μl)	1-20μl
	2M $CaCl_2$	18μl
	Nuclease-free water	to 150μl
Tube 2	2X HBS	150μl

3. The solution in tube 1 was slowly added drop-wise to the solution in tube 2 while air was bubbled through the solution in tube 2 with a pipette. The solution mix was incubated at room temperature for 30 minutes, then added to the 3ml of growth medium in each 6-well. Cells were incubated in a humidified incubator at 37°C/5% CO_2 for 24 hours.

4. Growth medium was removed and cells were washed twice with PBS_A and either harvested for analysis of gene expression or growth medium was replaced and cells were cultured as desired.

LipofectAMINE™, a cationic lipid mediated transfection protocol:

LipofectAMINE reagent is a 3:1 (w/w) formulation of the polycationic lipid DOSPA and the neutral lipid DOPE. The use of cationic lipids for DNA transfer was first reported by(Felgner et al., 1987).

1. Cultured cells were seeded at 1-2x10^5/well in a 6-well culture plate. Cells were left to adhere for 24-36 hours or were cultured until 60% confluency.

2. For each transfection two 1.5ml microcentrifuge tubes were set up as follows;

		per 6-well
Tube A	DNA (1μg/μl)	1-4μl
	Serum/antibiotic-free medium	100μl
Tube B	LipofectAMINE	12μl
	Serum/antibiotic-free medium	100μl

3. Both solutions were combined with gentle mixing and incubated at room temperature for 45 minutes. Cells were rinsed once with 2ml of serum-free growth medium.

4. 800µl of serum-free growth medium was added to each tube containing the DNA-liposome complexes and mixed gently. Culture medium was aspirated from the cultured cells and the diluted complex solution was overlayed and incubated at 37°C 5% CO_2 for 5 hours.

5. Following incubation with DNA-liposome complexes 1ml of growth medium containing 20% (v/v) FCS was added and incubation was continued. At 24 hours post-transfection cells were harvested for analysis of gene expression or growth medium was replaced and cells were cultured as desired.

SuperFect™ mediated transfection:

SuperFect reagent is a specifically designed activated polyamidoamine dendrimer and was used as an alternative protocol for cells which are sensitive to transfection by classical cationic lipid methods.

1. Cultured cells were seeded at 1-2x10^5/well in a 6-well culture plate. Cells were left to adhere for 24-36 hours or were cultured until 60% confluency.

2. 2-20µg of DNA was diluted with cell growth medium containing no serum, proteins or antibiotics to a total volume of 100µl and mixed. 15µl of SuperFect transfection reagent was added to the DNA solution and was mixed by vortexing briefly. Samples were incubated at room temperature for 10 minutes.

3. Growth medium was aspirated from the culture dish and cells were washed once with 4ml of PBS_A. 1ml of normal cell growth medium was added to each reaction tube and was mixed by pipetting up and down twice.

4. The contents of each reaction tube was pipetted to the relevant well of the culture dish and cells were incubated at 37°C 5% CO_2 for 3 hours. Growth medium was removed by gentle aspiration and cells were washed once with PBS_A and was replaced with fresh growth medium. Cells were cultured for 24 hours and either harvested for analysis of gene expression or maintained in culture for as long as desired.

Selection of stable clones expressing a transfected gene:

Stable transfection results when cells incorporate DNA into their chromosomal DNA or maintain it as an episome. Selection of stable transfected cells is carried out with a suitable selective pressure such as antibiotic resistance. Addition of the antibiotic Geneticin (G418) to growth medium allows selection of cells expressing the bacterial gene which confers resistance to Geneticin. Stable, single clones were isolated by plating out a serial dilution of cells to less than 0.5 cells per well of a 24-well tissue culture plate and grown in medium containing 1mg ml^{-1} Geneticin (Gibco-BRL).

CHAPTER 4

The expression of c-*fms* and expression of associated intracellular signalling proteins in unstimulated and stimulated BAC1.2F5 macrophages.

4.0.0. Introduction

M-CSF stimulates the proliferation and differentiation of macrophages and their precursors (Stanley et al., 1994). The receptor for M-CSF, the c-*fms* gene product, is a protein tyrosine kinase expressed mainly by cells of the monocyte-macrophage lineage. Expression of both M-CSF and its receptor by the same cell can establish an autocrine signalling loop resulting in uncontrolled proliferation (Stanley et al., 1994). On binding of M-CSF, the c-*fms* receptor becomes transphosphorylated on tyrosine within the cytoplasmic domain. These autophosphorylation sites create potential high affinity binding sites for SH2-domain containing proteins and in turn induce the phosphorylation of several of these cellular proteins on tyrosine (Hamilton, 1997). To date, several tyrosine autophosphorylation sites have been identified on c-*fms* including Y^{699}, Y^{708}, Y^{723} in the kinase insert region, Y^{809} in the tyrosine kinase domain, and Y^{561} in the juxtamembrane region (Reedijk et al., 1992; Tapley et al., 1990; van der Geer and Hunter, 1990). The cellular proteins Grb2 (van der Geer and Hunter, 1993), PI 3-kinase (Varticovski et al., 1989), STAT1 (Novak et al., 1996) and Src (Alonso et al., 1995) have been shown to bind to these tyrosine phosphorylation sites, respectively. Although association of these intracellular proteins has been clearly demonstrated *in vitro*, there is no clear consensus on what cellular responses they mediate or how they interact with each other at the receptor complex. To date the published data has only demonstrated that there is a need for a better cell based model system for characterisation of c-*fms* signalling. At the very least c-*fms* signalling must be studied within the context of the haematopoietic lineage to allow clear interpretation of the data obtained from the particular cell lineage studied.

As a model system with which to study PI 3-kinase activity during c-*fms* signalling, the cloned SV40-immortalised mouse macrophage cell lines BAC1.2F5 was chosen

based on a number of criteria. BAC1.2F5 cells resemble primary macrophages in their requirement for M-CSF for both survival and proliferation. BAC1.2F5 cells have previously been well characterised, expressing c-*fms* at high levels and responding to M-CSF with rapid morphological changes including membrane ruffling, cytoskeletal reorganisation and vesicle formation as well as M-CSF stimulation of survival and proliferation (Boocock et al., 1989).

The overall aim of Chapter 4 was to characterise the BAC1.2F5 cell line as a model for M-CSF stimulated, c-*fms*-mediated responses. The specific aims of this chapter were; (a) determine the expression profile of c-*fms* in M-CSF stimulated or quiescent BAC1.2F5 cells, (b) determine the tyrosine phosphorylation state of c-*fms* during M-CSF stimulation and (c) determine which PI 3-kinase subunit isoforms were expressed in BAC1.2F5 cells. In addition the expression of intracellular signalling molecules, previously shown or thought to be involved in c-*fms* signalling were also investigated.

4.1.0. BAC1.2F5 cells express the c-*fms*.

The expression of c-*fms* and, for comparison, the PDGFr was studied by Fluorescence Activated Cell Sorting (FACS) analysis (chapter 3.3.6.). BAC1.2F5 and U937 cells, which resemble undifferentiated macrophage progenitors, were cultured in the absence of growth factors for 24 hours. Cells were then cultured for a further 24 hours in either recombinant murine M-CSF (100ng ml^{-1}, R&D Systems) or recombinant $PDGF_{\alpha/\beta}$ (25ng ml^{-1}, Sigma). Cells were immunolabelled with antibodies raised against the murine M-CSF (Santa Cruz) and PDGF (Sigma) receptors and analysed by FACS. The results show that U937 cells express the M-CSF and PDGF receptors at constitutively high levels and treatment of U937 cells with either growth factor had no effect on levels of receptor expression (Fig. 4.1.1.).

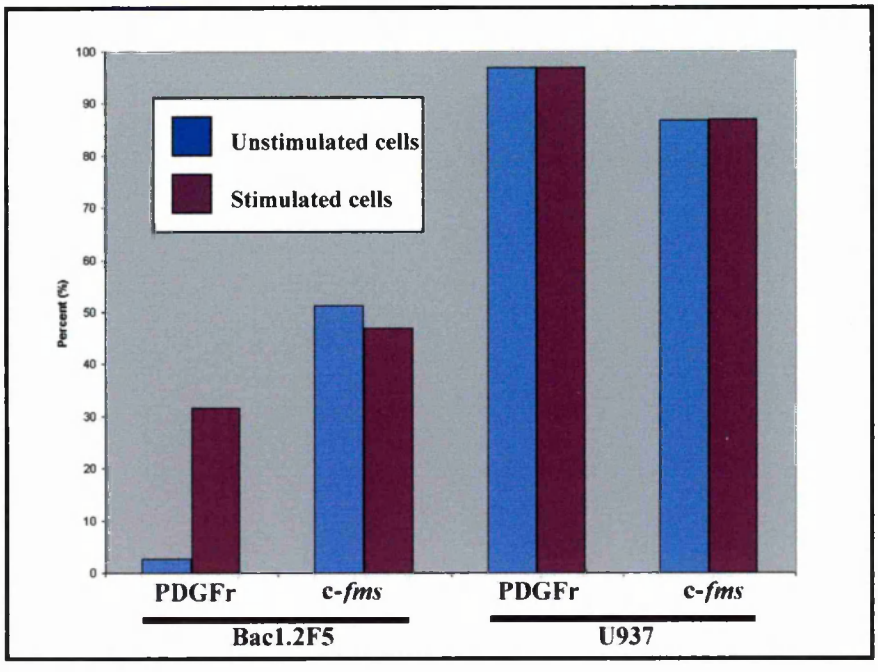


Fig. 4.1.1. FACS analysis of the expression of c-*fms* and PDGFr on BAC1.2F5 and U937 cells. U937 and BAC1.2F5 cells were cultured in the absence of growth factors for 24 hours prior to culture in the presence of either recombinant PDGFβ (25ng ml^{-1}, Sigma) or recombinant murine M-CSF (100ng ml^{-1}) for a further 20 hours. $2x10^6$ cells were harvested, BAC1.2F5 cells were dissociated from plastic with Trypsin/0.02% EDTA (Imperial labs.), and labelled with either anti-PDGFr mAb (Sigma) or anti-c-*fms* mAb (Santa Cruz). A FITC labelled goat anti-mouse (Amersham) second antibody was used to detect cells labelled with primary antibody. Cells were analysed by FACS analysis on a FACScan (Beckton-Dickinson).

BAC1.2F5 cells express the c-*fms* (≈ 50% cells) but only a small proportion of the population sampled expressed the PDGFr (< 5%) (Fig. 4.1.1.). Treatment of BAC1.2F5 cells with rPDGFα/β up-regulated PDGFr expression (≈ 30 %) (Fig. 4.1.1.). There appears to be a slight reduction in c-*fms* expression following treatment with rmM-CSF, which may be due to rapid receptor degradation (Boocock et al., 1989).

4.2.0. Western blot analysis of c-*fms*

Western blot analysis (chapter 3.3.1.) of whole cell lysates obtained from BAC1.2F5 cells indicated that two receptor species were present: a 130kDa immature form and a predominant, 165kDa mature form (Fig. 4.2.1.). A 30 minute treatment of BAC1.2F5 cells with M-CSF resulted in a slight reduction in the detected levels of the 130kDa immature form of the receptor and a dramatic decrease in the levels of the mature 165kDa form (Fig. 4.2.1.).

Stimulation of BAC1.2F5 cells with M-CSF resulted in a rapid increase in the detectable levels of protein phosphorylation on tyrosine (Fig. 4.2.2.). It has previously been demonstrated that stimulation of BAC1.2F5 cells at 4°C enhances detection of tyrosine phosphorylated receptor and proteins which are tyrosine phosphorylated in response to c-*fms* activation (Baccarini et al., 1991). Therefore incubation of cells at 4°C was used to retard the usually rapid dephosphorylation of c-*fms* and other proteins phosphorylated in response to M-CSF stimulation of BAC1.2F5 cells.

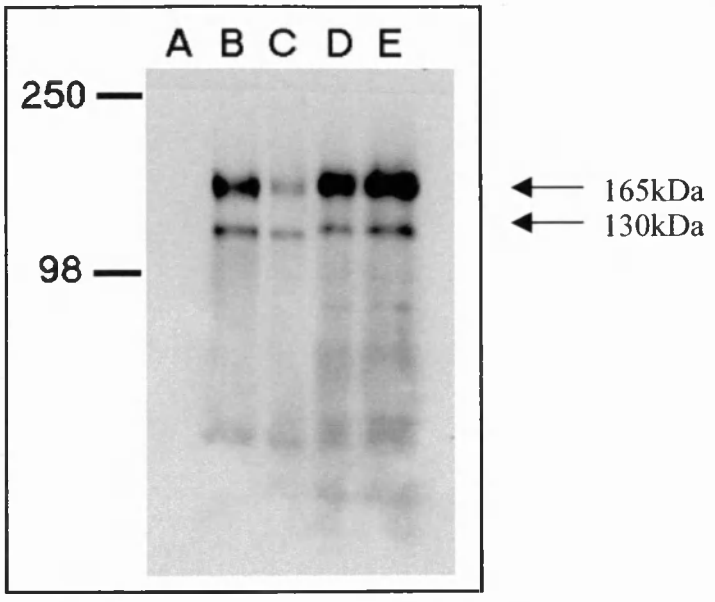


Fig. 4.2.1. SDS-PAGE analysis of total protein extracts immunoblotted for c-*fms*. Total cell lysate proteins were resolved by 7.5% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-c-*fms* pAb (Santa Cruz). Lysate from stimulated A431 (A), unstimulated BAC1.2F5 cells cultured for 30′ at 37°C (B), BAC1.2F5 cells stimulated for 30′ at 37°C (C), BAC1.2F5 cells stimulated for 2′ at 37°C (D) and BAC1.2F5 cells stimulated for 30′ at 4°C (E) were loaded onto the gel.

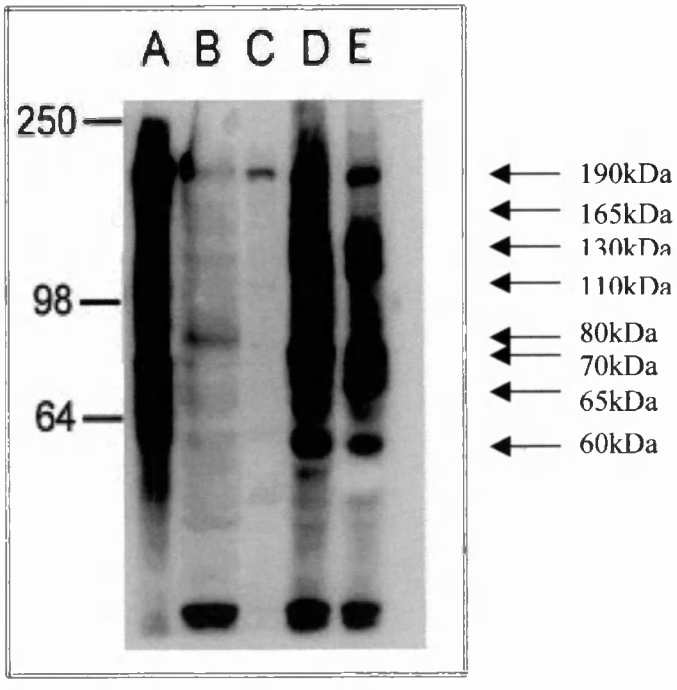


Fig. 4.2.2. SDS-PAGE analysis of total protein extracts immunoblotted for tyrosine phosphorylated proteins. Approximately 10μg of total cell lysate proteins were resolved by 7.5% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-phosphotyrosine mAb (Transduction labs.). Lysate from stimulated A431 (A), unstimulated U937 (B), unstimulated BAC1.2F5 (C), BAC1.2F5 stimulated for 30′ at 4°C (D) and BAC1.2F5 stimulated for 30′ at 37°C (E) were loaded onto the gel.

In quiescent BAC1.2F5 cells few, if any, tyrosine phosphorylated proteins were detected in whole cell lysate (Fig. 4.2.2., lane C). Only a 190kDa protein appeared to be phosphorylated in unstimulated cells (Fig. 4.2.2., lane C). However, stimulation with M-CSF at 4°C had a substantial effect on the number of tyrosine phosphorylated proteins detected (Fig. 4.2.2., lane D). Various tyrosine phosphorylated bands were detected in whole cell lysate at 190kDa, 165kDa, 120kDa, 110kDa, 80kDa, 70kDa, 65kDa and 60kDa (Fig. 4.2.2., lane D). Stimulation of BAC1.2F5 cells for 30 minutes at 37°C resulted in detection of fewer phosphorylated bands, but proteins at 190kDa, 130kDa, 110kDa, 80kDa, 70kDa and 60kDa were present (Fig. 4.2.2., lane E). A tyrosine phosphorylated band of 165 kDa was detected from stimulated cells which migrated on SDS-PAGE at an equivalent M_r to the mature form of c-*fms* (Fig. 4.2.2., lanes D and E). The absence of this band in unstimulated cell lysate (Fig. 4.2.2., lane C) suggests that c-*fms* is tyrosine phosphorylated in response to M-CSF (Fig. 4.2.2., lanes D and E). The identity of this band was investigated further and is described in the next set of experiments.

Western blot analysis of c-*fms* immunoprecipitated (chapter 3.3.2.) from BAC1.2F5 cells was carried out from lysates obtained from either parental BAC1 or BAC1.2F5 cells. Immunoprecipitated protein was analysed by western blot and probed with an antibody against the c-*fms* receptor (Fig. 4.2.3.). The mature 165kDa receptor form was not detected in the parental BAC1 cell line, although the immature 130kDa isoform (lower arrow) was immunoprecipitated from BAC1 cells (Fig. 4.2.3., lane C). In contrast both receptor isoforms were immunoprecipitated from unstimulated BAC1.2F5 lysate and the mature isoform appeared to be the predominantly expressed protein in quiesced cells (Fig. 4.2.3., lane G). Treatment of cells with M-CSF at 37°C resulted in reduced detection of mature receptor by 2 minutes (Fig. 4.2.3., lane D)

which was more pronounced at 30 minutes (Fig. 4.2.3., lane F), but had no affect on detection of the immature c-*fms* isoform (Fig. 4.2.3., lanes D+F). Stimulation of BAC1.2F5 cells at 4°C blocked degradation of the immunoprecipitated, mature receptor isoform.

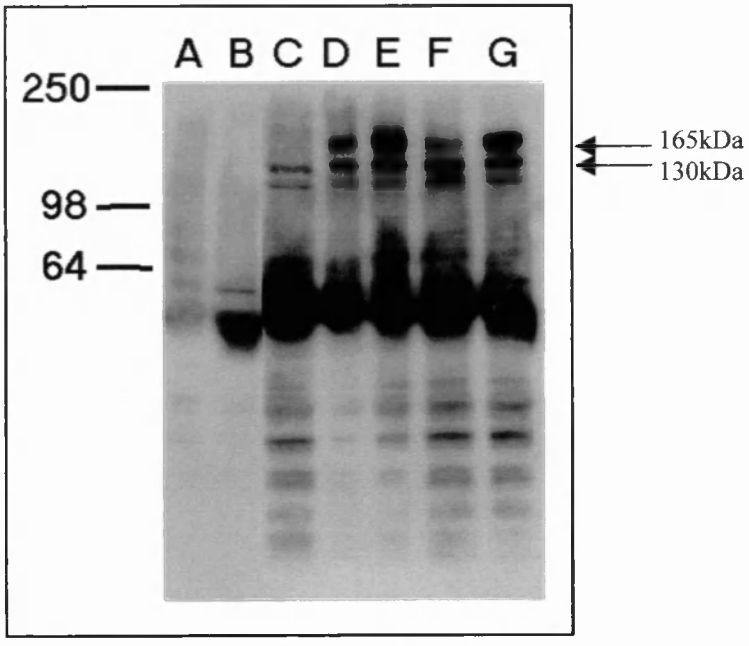


Fig. 4.2.3. SDS-PAGE analysis of protein immunoprecipitated from BAC1 parental or BAC1.2F5 cell line immunoblotted for c-*fms*. Immunoprecipitated proteins were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-c-*fms* pAb (Santa Cruz). Lysate from stimulated A431 (A) and NIH 3T3 (B) cell lines were run as controls. Lanes C to G contain anti-c-*fms* immunoprecipitates from; BAC1 parental cells with anti-c-*fms* antibody (C), BAC1.2F5 cells stimulated for 2′ at 37°C (D), BAC1.2F5 cells stimulated for 30′ at 4°C (E), BAC1.2F5 cells stimulated for 30′ at 37°C (F) and unstimulated BAC1.2F5 cells (G).

4.3.0. Association of tyrosine phosphorylated proteins with c-*fms*

Proteins that were associated with immunoprecipitated c-*fms* were probed with an antibody against phosphotyrosine (Fig. 4.3.1.). Numerous phosphorylated proteins were detected including two bands at 130kDa and 165kDa which co-migrated at a comparable M_r to the c-*fms* isoforms identified in Fig. 4.2.3. In addition, phosphorylated proteins of 55kDa, 60kDa, 80kDa, 115kDa, 190kDa, and multiple

proteins <40kDa were detected in unstimulated and stimulated BAC1.2F5 lysate (Fig. 4.3.1.). In unstimulated cells, the 165kDa c-*fms* isoform contained a low level of tyrosine phosphorylation and co-precipitated proteins with molecular weights of 190kDa, 115kDa and several smaller phosphoproteins below 40kDa (Fig. 4.2.4., lane D).

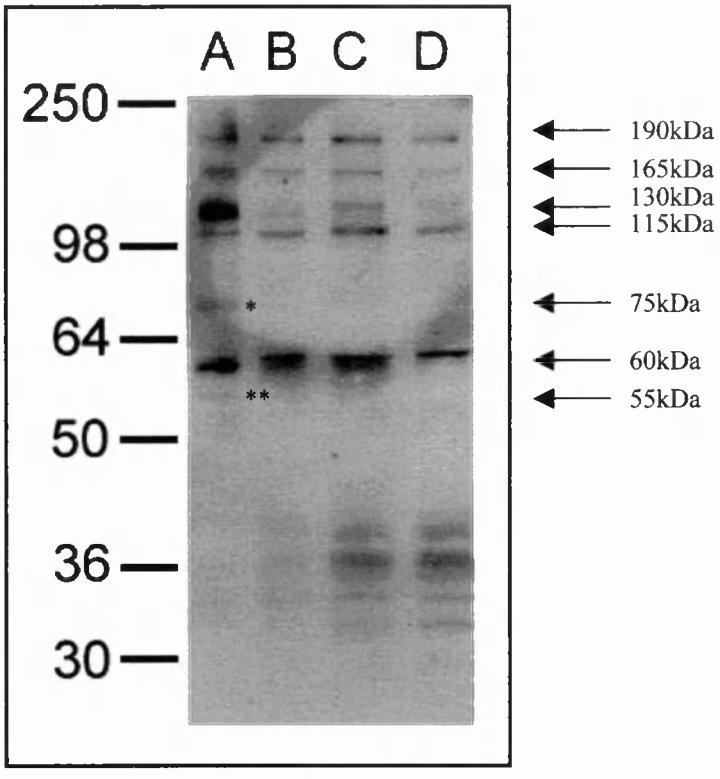


Fig. 4.3.1. SDS-PAGE analysis of proteins co-immunoprecipitated with c-*fms* from BAC1.2F5 cell lysates and immunoblotted for tyrosine phosphorylation. Immunoprecipitated proteins were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-phosphotyrosine mAb (Transduction Labs.). Lanes A to D contain proteins co-immunoprecipitated from BAC1.2F5 cells stimulated for 2′ at 37°C (A), stimulated for 30′ at 4°C (B), stimulated for 30′ at 37°C (C) and unstimulated (D).

Cells which have been stimulated for 30 minutes at 37°C (Fig. 4.3.1., lane C) and at 4°C (Fig. 4.3.1., lane B) showed increased levels of tyrosine phosphorylation of the 165kDa receptor isoform and co-immunoprecipitated phosphoproteins with molecular weights of 190kDa, 115kDa and several bands below 40kDa, similar to unstimulated cells. Interestingly, stimulation of BAC1.2F5 cells for 2 minutes at 37°C results in

higher levels of tyrosine phosphorylation of both c-*fms* isoforms, particularly the 130kDa isoform compared to unstimulated cells (Fig. 4.3.1., lane A). At 2 minutes two additional phosphorylated proteins were detected at 75kDa and 55kDa (indicated by * and **, respectively) that were not detected in unstimulated cells or cells stimulated for 30 minutes at either 37°C or 4°C (Fig. 4.3.1., lane A).

4.4.0. Expression of PI 3-kinase by BAC1.2F5 cells.

Western blot analysis of BAC1.2F5 lysates was carried out with antibodies raised against the α,β and γ isoforms of the p85 regulatory subunit of PI 3-kinase. It was observed that BAC1.2F5 cells express the p85α subunit (Fig. 4.4.1.), but do not express either p85β (Fig. 4.4.2.) or the 55kDa, p85γ subunit (Fig. 4.4.3.) of PI 3-kinase.

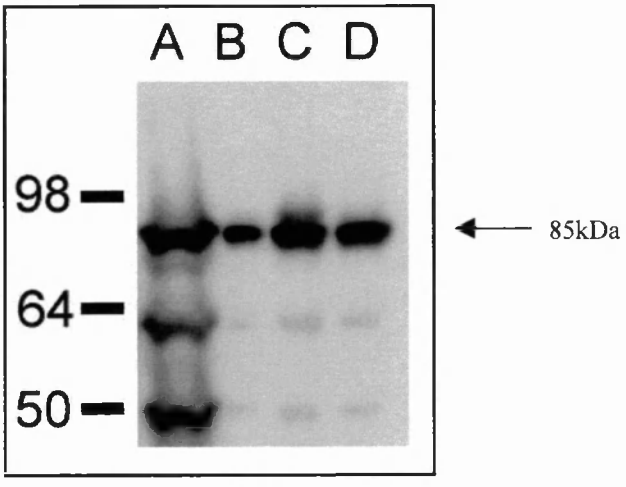


Fig. 4.4.1. SDS-PAGE analysis of total protein extracts immunoblotted for p85α. Total cell lysates were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-p85α pAb (Transduction labs.). Lysate from stimulated A431 (A), unstimulated BAC1.2F5 (B), BAC1.2F5 stimulated for 30′ at 4°C (C) and BAC1.2F5 stimulated for 30′ at 37°C (D) were loaded onto the gel.

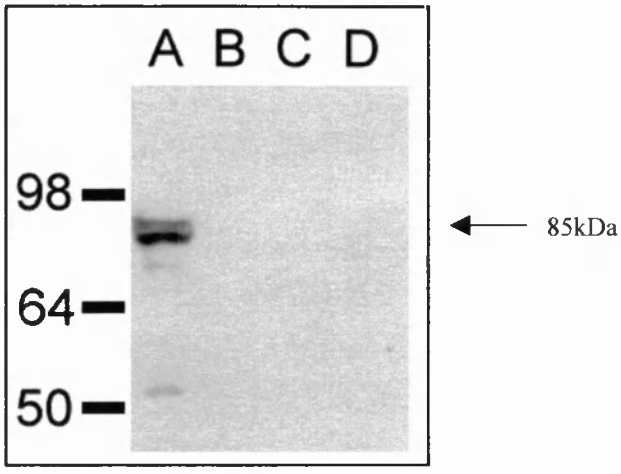


Fig. 4.4.2. SDS-PAGE analysis of total protein extracts immunoblotted for p85β. Total cell lysates were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-p85β mAb (Serotec). Lysate from stimulated A431 (A), unstimulated BAC1.2F5 (B), BAC1.2F5 stimulated for 30′ at 4°C (C) and BAC1.2F5 stimulated for 30′ at 37°C (D) were loaded onto the gel.

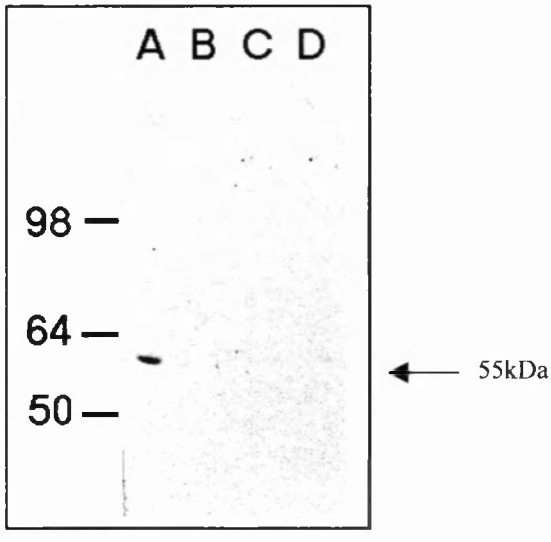


Fig. 4.4.3. SDS-PAGE analysis of total protein extracts immunoblotted for p85γ. Total cell lysates were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-p85γ mAb (Serotec). Lysate from human fibroblasts (A), unstimulated BAC1.2F5 (B), BAC1.2F5 stimulated for 30′ at 4°C (C) and BAC1.2F5 stimulated for 30′ at 37°C (D) were loaded onto the gel.

Analysis of BAC1.2F5 expression of the catalytic subunit of PI 3-kinase revealed that BAC1.2F5 cells expressed the p110α subunit (Fig. 4.4.4.) and the G-protein associated p110γ subunit (Fig. 4.4.6.), but did not appear to express the p110β subunit (Fig. 4.4.5.).

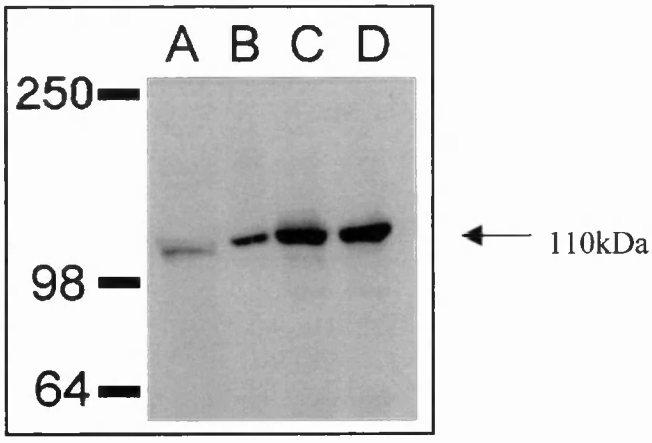


Fig. 4.4.4. SDS-PAGE analysis of total protein extracts immunoblotted for p110α. Total cell lysates were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-p110α pAb (Santa Cruz). Lysate from stimulated A431 (A), unstimulated BAC1.2F5 (B), BAC1.2F5 stimulated for 30′ at 4°C (C) and BAC1.2F5 stimulated for 30′ at 37°C (D) were loaded onto the gel.

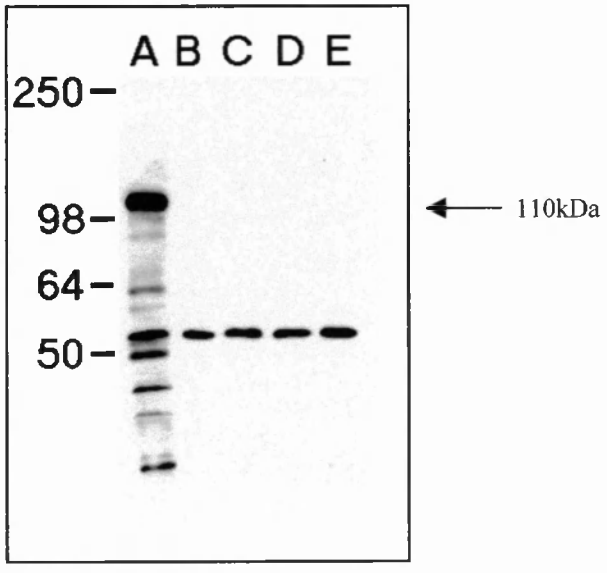


Fig. 4.4.5. SDS-PAGE analysis of total protein extracts immunoblotted for p110β. Total cell lysates were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-p110β pAb (Santa Cruz). Lysate from stimulated A431 (A), unstimulated BAC1.2F5 (B), BAC1.2F5 stimulated for 30′ at 4°C (C) and BAC1.2F5 stimulated for 2′ at 37°C (D) and 30′ at 37°C (E) were loaded onto the gel.

Unlike the c-*fms* protein product, the PI 3-kinase subunits, p85α and p110α were not degraded following M-CSF stimulation. Any apparent difference in the detected levels of p85 or p110 subunits following treatment with M-CSF were due to unequal protein loading, as confirmed by Coomassie blue staining of parallel gels (data not shown).

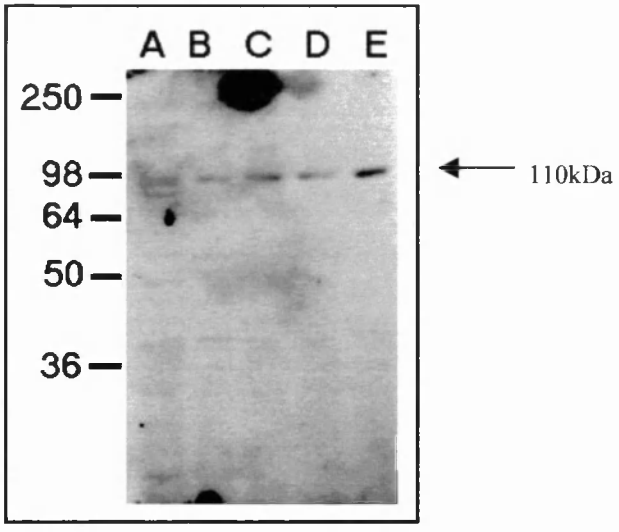


Fig. 4.4.6. SDS-PAGE analysis of total protein extracts immunoblotted for p110γ. Total cell lysates were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-p110γ pAb (Santa Cruz). Lysate from stimulated A431 (A), unstimulated BAC1.2F5 (B), BAC1.2F5 stimulated for 30′ at 4°C (C), BAC1.2F5 stimulated for 2′ at 37°C (D) and 30′ at 37°C (E) were loaded onto the gel.

4.5.0. Western blot analysis of c-*fms*-associated signalling proteins

It has previously been demonstrated that numerous intracellular signalling proteins bind to or are associated with c-*fms* including c-*src*, Grb2, STAT-1, PI 3-kinase, Sos1, Shc, SHIP, and c-*cbl*. To characterise the signalling proteins expressed in BAC1.2F5 cells western blot analysis was carried out on BAC1.2F5 whole cell lysate with antibodies raised against various intracellular proteins implicated in c-*fms* signalling including c-*src*, Grb2, Sos1, c-*cbl* and Shc.

Various combinations of these proteins exist as complexes in monocytic cells and binding sites for the adapter proteins Grb2 and Shc have been mapped to SH2 and PTB binding domains on the cytoplasmic tail of c-*fms* (Lioubin et al., 1994). Analysis of BAC1.2F5 whole cell lysates has shown that this cell line expresses Grb2 (24 kDa), Sos1 (170kDa), Shc (66, 52 and 46kDa), c-src (55kDa) and c-cbl (120kDa) (Figs. 4.5.1-5.). There is no down-regulation of expression of these proteins following M-CSF stimulation, any apparent differences are due to unequal protein loading as confirmed by Coomassie Blue staining of parallel gels (data not shown).

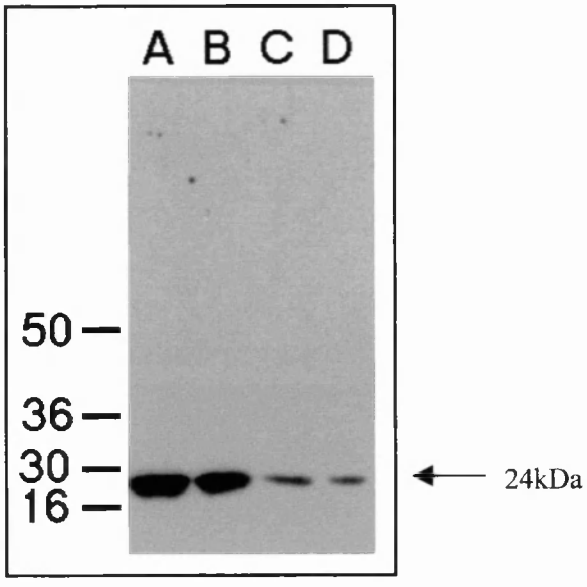


Fig. 4.5.1. SDS-PAGE analysis of total protein extracts from BAC1.2F5 cells immunoblotted for Grb2. Proteins were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-Grb2 mAb (Transduction Labs.). Lanes A and B contain lysate obtained from stimulated A431 and NIH 3T3 cells, respectively. Lanes C and D contain lysate obtained BAC1.2F5 cells quiesced for 24 hours in the absence of M-CSF then stimulated for 30 minutes at 4°C, respectively.

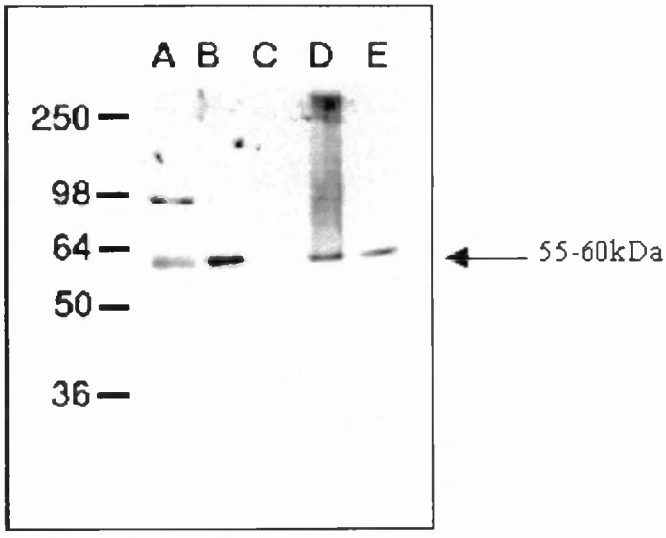


Fig. 4.5.2. SDS-PAGE analysis of total protein extracts from BAC1.2F5 cells immunoblotted for c-*src*. Proteins were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-c-Src pAb (Santa Cruz). Lysate from stimulated A431 cells (A), unstimulated BAC1.2F5 cells (B), BAC1.2F5 cells stimulated for 30′ at 4°C (C), BAC1.2F5 cells stimulated for 2′ at 37°C (D) and BAC1.2F5 cells stimulated for 30′ at 37°C (E) were loaded onto the gel.

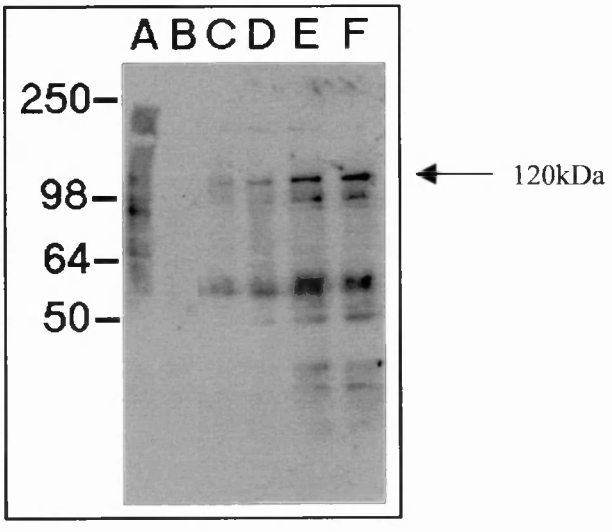


Fig. 4.5.3. SDS-PAGE analysis of total protein extracts from BAC1.2F5 cells immunoblotted for c-*cbl*. Proteins were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-c-Cbl pAb (Santa Cruz). Lysate from stimulated A431 (A) and NIH 3T3 (B) cell lines were run as controls. Lanes C, D, E and F contain lysate from BAC1.2F5 cells which have been stimulated for 2′ at 37°C, stimulated for 30′ at 4°C, stimulated for 30′ at 37°C and unstimulated, respectively.

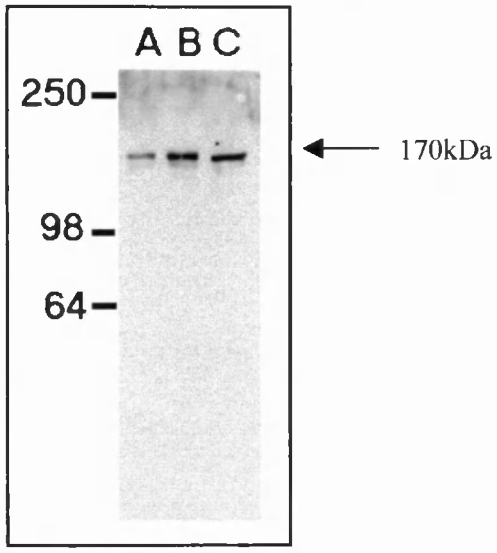


Fig. 4.5.4. SDS-PAGE analysis of total protein extracts from BAC1.2F5 cells immunoblotted for Sos1. Proteins were resolved by 7.5% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-Sos1 mAb (Transduction Labs.). Lane A contains lysate from stimulated A431 cells. Lanes B and C contain lysates from BAC1.2F5 cells quiesced for 24 hours in the absence of M-CSF then stimulated for 30 minutes at 37°C, respectively.

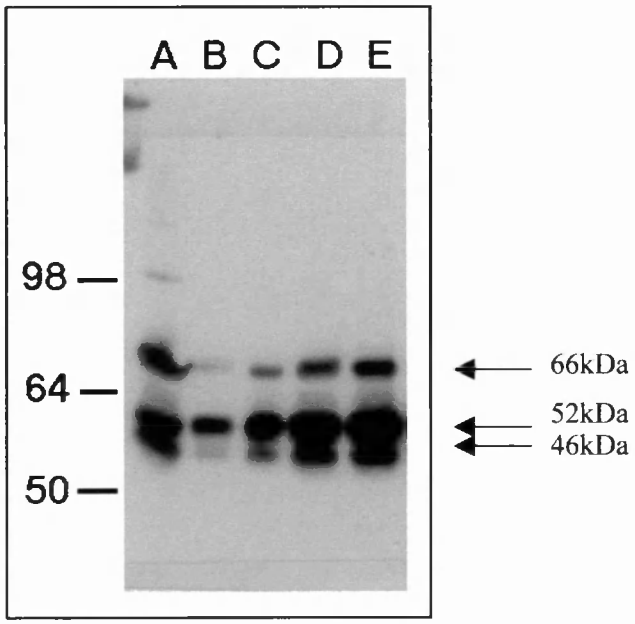


Fig. 4.5.5. SDS-PAGE analysis of total protein extracts from BAC1.2F5 cells immunoblotted for Shc. Proteins were resolved by 10% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-Shc pAb (Transduction Labs.). Lane A contains lysate from human fibroblasts, lanes B, C, D and E contain lysates obtained from BAC1.2F5 cells quiesced for 24 hours in the absence of M-CSF, stimulated for 2 minutes at 37°C, stimulated for 30 minutes at 37°C and stimulated for 30 minutes at 4°C, respectively.

4.6.0. M-CSF stimulated PI 3-kinase activity in BAC1.2F5 macrophages

The effect of M-CSF stimulation on PI 3-kinase activity in BAC1.2F5 cells was investigated by an *in vitro* PI 3-kinase activity assay (chapter 3.3.9.). PI 3-kinase activity was immunoprecipitated from BAC1.2F5 cells with an anti-p85α antibody immobilised on agarose beads. The immunoprecipitated PI 3-kinase activity was quantified by its ability to catalyse the incorporation of a γ-phosphate from ^{32}P-radiolabelled ATP onto either PI or PI_4P to produce PI_3P and $PI_{3,4}P_2$, respectively. Radiolabelled lipids were resolved by thin layer chromatography and compared to unlabelled standard. Fig. 4.6.1. is an example of a PI 3-kinase assay carried out over a 20 minute time-course following M-CSF stimulation of BAC1.2F5 cells.

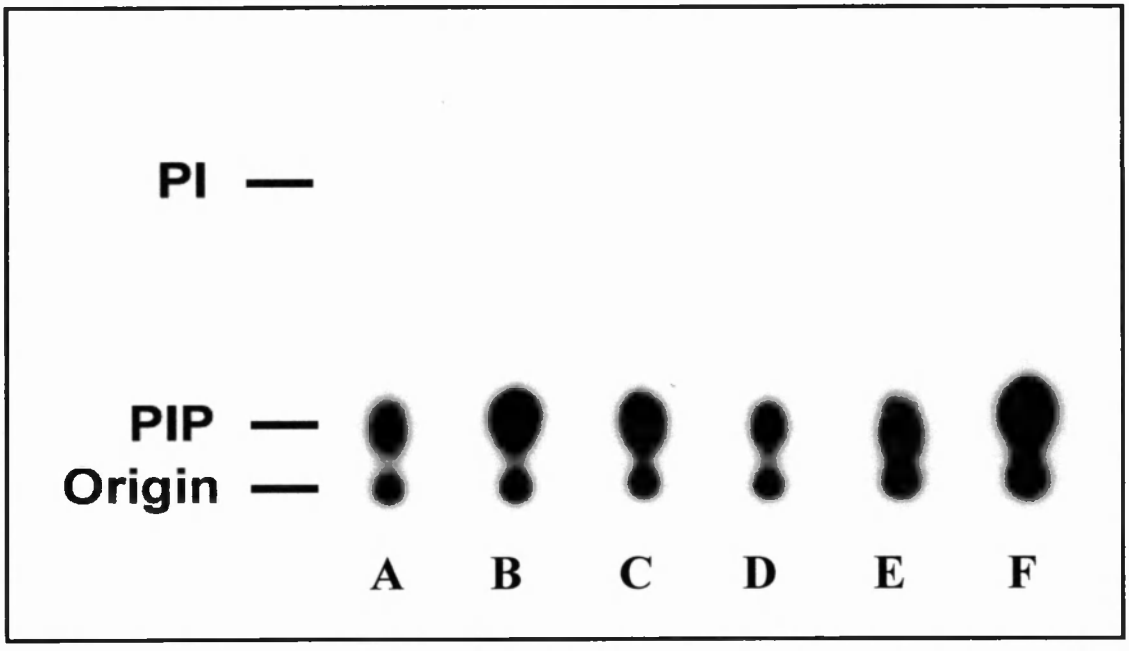


Fig. 4.6.1. In vitro PI 3-kinase activity of anti-p85α immunoprecipitates from BAC1.2F5 cells. Phospholipids obtained from the *in vitro* PI 3-kinase assays were loaded onto a thin layer chromatography plates and run until all lipids had been separated. Lipid standards were visualised by exposure to iodine crystals and are indicated. Each lane corresponds to PI 3-kinase activity immunoprecipitated from either unstimulated BAC1.2F5 cells (A), BAC1.2F5 cells stimulated with 50ng ml^{-1} M-CSF for 5 minutes (B), 10 minutes (C) 15 minutes (D) and 20 minutes (E) at 37°C. Lane F corresponds to PI 3-kinase activity immunoprecipitated from BAC1.2F5 cells stimulated for 20 minutes at 4°C.

The results obtained for the PI 3-kinase assays represented in Fig. 4.6.1. were quantified on an InstantImager™ and are presented in Fig. 4.6.2. M-CSF stimulated a 3-fold increase in PI 3-kinase activity after 5 minutes which was still present by 10 minutes but had returned to basal levels after 15 minutes (Fig. 4.6.2.). A slight increase was observed at 20 minutes, but this was only a 1.5-fold increase over basal levels (Fig. 4.6.2.). Incubation at 4°C for 20 minutes resulted in a 5-fold increase in PI 3-kinase activity over basal levels (Fig. 4.6.2.). Incubation of cells at 4°C appears to maintain PI 3-kinase activation possibly because 4°C incubations have been reported to slow receptor dephosphorylation (Fig. 4.6.2.).

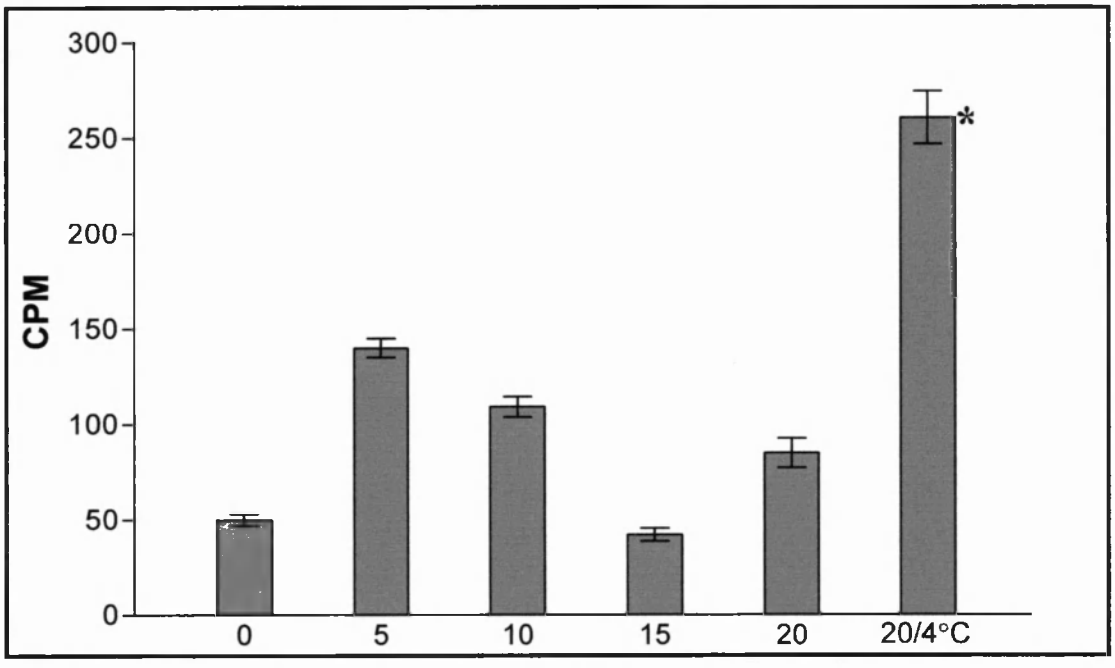


Fig. 4.6.2. Quantification of *in vitro* PI 3-kinase activity immunoprecipitated from BAC1.2F5 cells stimulated with M-CSF measured by PI_3P formation. Radiolabelled PI_3P was quantified on a Phosphoimager (Packard) and expressed as counts per minute, (cpm). BAC1.2F5 cells were incubated with 50ng ml^{-1} M-CSF from 0 to 20 minutes at 37°C. * indicates cells incubated at 4°C. Error bars indicate SEM *(n=3)*.

To demonstrate that the immunoprecipitated PI 3-kinase activity was sensitive to wortmannin or LY294002, BAC1.2F5 cells were pre-incubated with both inhibitors prior to stimulation with M-CSF. Following treatment with inhibitors, BAC1.2F5

cells were incubated with 50ng ml^{-1} M-CSF for 10 minutes before immunoprecipitation of PI 3-kinase with an anti-p85α antibody immobilised on protein-A agarose beads. Fig. 4.6.3. represents a PI 3-kinase assay carried out 10 minutes following M-CSF stimulation of BAC1.2F5 cells.

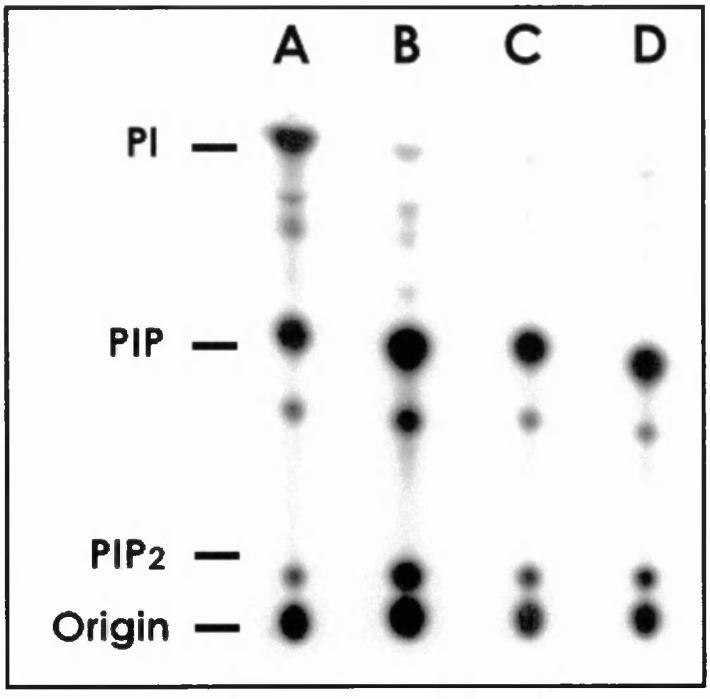


Fig. 4.6.3. In vitro PI 3-kinase activity of anti-p85α immunoprecipitates from BAC1.2F5 cells. Phospholipids obtained from the *in vitro* PI 3-kinase assays were loaded onto a thin layer chromatography plates and run until all lipids had been separated. Lipid standards were visualised by exposure to iodine crystals and are indicated. Each lane corresponds to PI 3-kinase activity immunoprecipitated from either unstimulated BAC1.2F5 cells (A), BAC1.2F5 cells stimulated with M-CSF for 10 minutes (B), and stimulated cells pre-incubated with 30nM wortmannin (C) and 10μM LY294002 (D).

As observed in Fig. 4.5.1. M-CSF stimulation after 10 minutes at 37°C resulted in a 2-3-fold increase in PI 3-kinase activity, measured by PI_3P or $PI_{3,4}P_2$ formation (Fig. 4.6.4.). Incubation of BAC1.2F5 cells with the PI 3-kinase inhibitors wortmannin (30nM) and LY294002 (10μM) inhibited the M-CSF stimulated increase in PI 3-kinase activity to levels observed in unstimulated cells (Fig. 4.6.4.).

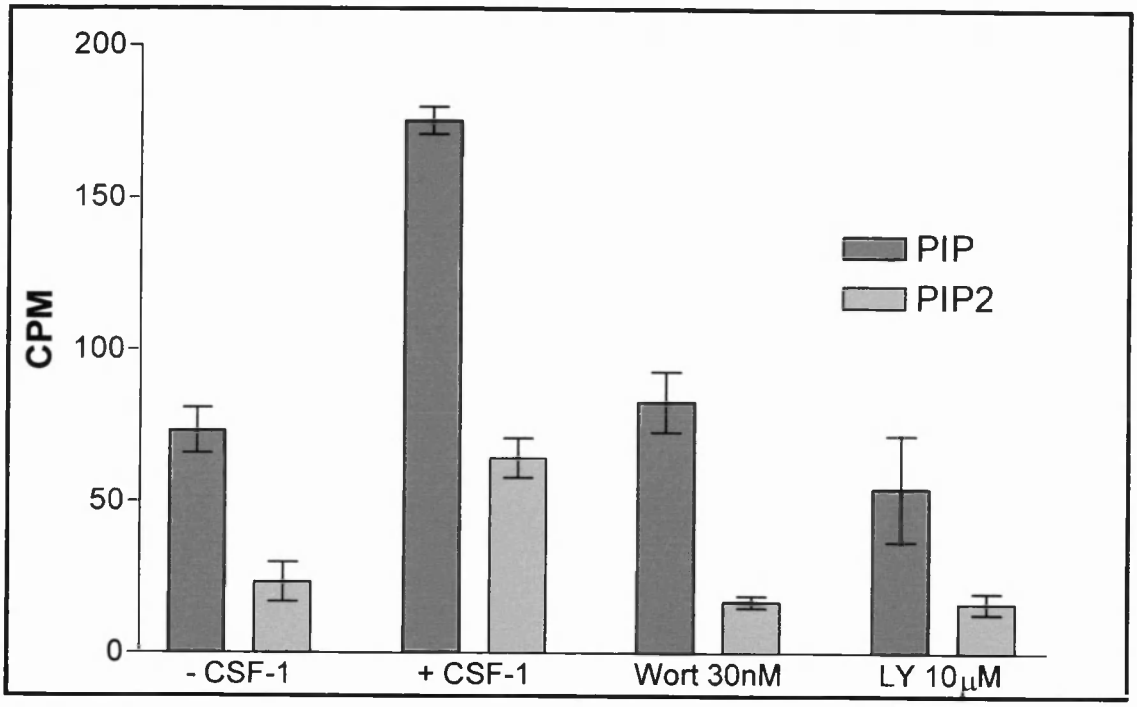


Fig. 4.6.4. Quantification of *in vitro* PI 3-kinase activity immunoprecipitated from BAC1.2F5 cells stimulated with M-CSF measured by PI_3P and $PI_{3,4}P_2$ formation. Radiolabelled PI_3P and $PI_{3,4}P_2$ were quantified on a Phosphoimager (Packard) and expressed as counts per minute, (cpm). BAC1.2F5 cells were incubated with 50ng ml^{-1} M-CSF for 15 minutes at 37°C. Cells were pre-incubated with PI 3-kinase inhibitors 15 minutes prior to stimulation with M-CSF. Error bars indicate SEM *(n=3)*.

4.7.0. Discussion

BAC1.2F5 cells require M-CSF for their survival and proliferation (Boocock et al., 1989). Analysis of BAC1.2F5 cells by FACS analysis showed that c-*fms* protein product is expressed on the cell surface of approximately 50% of BAC1.2F5 cells and on 90% on U937 positive control cells (Fig. 4.1.1.). This data may be interpreted such that BAC1.2F5 cultures contain a mixed population of macrophages with only a sub-population of cells actually expressing cell surface receptor, which may occur if there is a reversal in cell phenotype back to the parental BAC1 cell line, however this has not previously been reported. A more likely explanation is that BAC1.2F5 cells were routinely dissociated from tissue culture vessels by treatment with a trypsin/EDTA solution. Trypsin, which is a broad spectrum protease activity, may have enzymatically cleaved the extracellular domains of a proportion of cell surface receptors. This cleavage may have removed portions of the M-CSF receptor that contained the epitope required for antibody recognition. This may account, in part, for the lower levels of positive BAC1.2F5 cells detected by FACS analysis compared to the U937 cell controls which are not an adherent cell line (Fig. 4.1.1.).

Western blot analysis with antibodies specific for the c-*fms* demonstrated that BAC1.2F5 cells express both the immature and mature receptor isoforms (Fig. 4.2.1.) whereas the BAC1 parental cell line only express the immature receptor form (Fig. 4.2.3.). Following treatment with M-CSF for 30 minutes at 37°C there is less mature receptor detected in cells than in unstimulated controls (Fig. 4.2.1., lane C). However the rate of receptor internalisation and degradation can be reduced by incubating the cells with M-CSF at 4°C which was demonstrated by detected levels similar to unstimulated controls (Fig. 4.2.1., lane E). Lowering the incubation temperature also enhanced the detection of tyrosine phosphorylated proteins in whole cell lysates (Fig.

4.2.2). In unstimulated BAC1.2F5 cells receptor tyrosine phosphorylation was not detected in whole cell lysate (Fig. 4.2.2). However lack of detection was probably due to a lack of sensitivity since a basal level of tyrosine phosphorylation of both receptor isoforms was detected in immunoprecipitated receptor complexes (Fig. 4.3.1.). It appears that both receptor isoforms contain low levels of tyrosine phosphorylation. Phosphorylation of the mature receptor isoform may occur as a result of opportunistic dimerisation which activates the tyrosine kinase activity of c-*fms*, however this does not explain the phosphorylation observed in the immature receptor isoform. Fig. 4.2.3. illustrates degradation of receptor in cells stimulated with M-CSF for 30 minutes, which can be blocked by incubation at 4°C. Removal of M-CSF from culture medium has been reported to upregulate receptor expression in this cell line and mature receptor was readily detected (Boocock et al., 1989). Phosphorylation on tyrosine of proteins co-immunoprecipitated with c-*fms* also suggests that a low level of receptor tyrosine auto-phosphorylation occurs in unstimulated cells which again not present at detectable levels in whole cell lysate (Fig. 4.3.1.). Receptor activation stimulated the association and phosphorylation of various cellular proteins at 37°C, and although incubations at 4°C enhance the general levels of detectable tyrosine phosphorylation there was no change in the number of phosphorylated proteins detected (Fig. 4.3.1.). However, compared to the 30 minute incubations at either 37°C or 4°C, two additional tyrosine phosphorylated bands at 55kDa and 80kDa are present in immunoprecipitates from cells stimulated for 2 minutes at 37°C. These two phosphoproteins remain unidentified but it is possible that the 55kDa protein may be Shc.

Identification of the specific isoforms of PI 3-kinase present in BAC1.2F5 cells was carried out by western blot analysis. Using antibodies specific to the α, β and γ

isoforms of the p85 regulatory subunit of PI 3-kinase whole cell lysate from BAC1.2F5s were probed for p85 isoform expression. The only detectable isoform of p85 from BAC1.2F5 lysates was p85α and the levels of this species was not affected by c-*fms* activation with M-CSF (Fig. 4.4.1.). Western blot analysis failed to detect any p85β (Fig. 4.4.2.) or p85γ (Fig. 4.4.3.) in BAC1.2F5 cell lysates which were present in control cell lines. Immunoprobing of BAC1.2F5 whole cell lysate with antibodies specific to the α and β and γ isoforms of the p110 catalytic subunit of PI 3-kinase detected expression of the p110α and the p110γ isoforms (Fig. 4.4.4. and Fig. 4.4.6.). Expression levels of either p110α or p110γ were not affected by c-*fms* activation with M-CSF. Western blot analysis failed to detect any p110β (Fig. 4.4.5.) in BAC1.2F5 cell lysate which was present in control cell lines.

Analysis of PI 3-kinase activity following M-CSF stimulation of BAC1.2F5 cells revealed that PI3-kinase activity was increased 3-fold at 5 minutes (Figs. 4.5.1. and 4.5.2.). This suggests that activation of PI 3-kinase occurs rapidly during c-*fms* signalling in BAC1.2F5 cells. Incubation of BAC1.2F5s at 4°C further increased the detected levels of PI 3-kinase activity, which is probably because low temperature slowed down receptor dephosphorylation (Fig. 4.5.2.). The PI 3-kinase activity stimulated by M-CSF is most likely to be the classic p110α catalytic PI 3-kinase species as this was the predominant isoform. Although p110γ was detected this PI 3-kinase activity is regulated by G-protein coupled receptors rather than receptor tyrosine kinases. Incubation with wortmannin and LY294002 confirmed that the PI 3-kinase activity immunoprecipitated with anti-p85α antibody was sensitive to PI 3-kinase inhibitors (Figs. 4.5.3. and 4.5.4.). It appears that BAC1.2F5 cells express the

classical p85α/p110α class Ia PI 3-kinase activity which is inhibited by the fungal inhibitor wortmannin and the synthetic LY294002, two specific PI 3-kinase inhibitors. It must also be borne in mind that other p85 or p110 subunits may also be present but their detection may be limited by the unavailability or limits of currently available reagents.

CHAPTER 5

Effect of PI 3-kinase inhibitors wortmannin and LY294002 on M-CSF stimulated survival and proliferation of BAC1.2F5 macrophages.

5.0.0. Introduction

M-CSF is an important growth factor required for the survival, proliferation and differentiation of monocytes, macrophages, osteoclasts and their precursors. M-CSF binds with high affinity, $K_d = \approx 2x10^{-10}$, to murine c-*fms*, its specific cell-surface receptor which is found on virtually all mononuclear phagocytes (Das and Stanley, 1982; Welte et al., 1985).

It has been demonstrated that p85/p110α PI 3-kinase activity is required by the PDGF and EGF receptors for induction of DNA synthesis in quiescent NIH 3T3 cells (Roche et al., 1994; Singh et al., 1995). However, p85/p110α PI 3-kinase is not required for the propagation of a mitogenic signal by G-protein coupled receptors Bombesin and Lysophosphatidic Acid (LPA) nor appears to be required for M-CSF stimulated DNA synthesis in NIH 3T3 cells transfected with c-*fms* (Roche et al., 1994).

The requirement for PI 3-kinase activity during M-CSF stimulated proliferation of macrophages has not been investigated. However a number of M-CSF signalling studies have been carried out in c-*fms* transfected cell lines, with conflicting results. When phosphorylated, Tyr^{723} facilitates the binding of PI 3-kinase to the activated receptor. However, in NIH 3T3 fibroblasts the expression of c-*fms* which has had the kinase insert domain deleted does not significantly affect M-CSF induced cell proliferation, suggesting that tyrosine residues ($Tyr^{699,708,723}$), involved in PI 3-kinase and Grb2 binding, are not essential for the mitogenic signal (Reedijk et al., 1992; Shurtleff et al., 1990). Conversely, experiments performed in transfected rat fibroblasts (208F) demonstrate that mutation of Tyr^{721}Phe in murine c-*fms* resulted in a marked decrease in the ability of c-*fms* to bind the SH2 domain of the p85 subunit of PI 3-kinase with a concomitant loss in PI 3-kinase activity and proliferation (Reedijk

et al., 1992; van der Geer and Hunter, 1993). Much of the evidence that PI 3-kinase activity is not required is either based on studies of exogenous expression of intact c-*fms* in a cell background which is not related to the haematopoietic lineage. Therefore determining whether PI 3-kinase activity is required for M-CSF stimulated proliferation in a macrophage cell line may be useful since specific inhibitors of macrophage proliferation may be useful for treatment of auto-immune or hyperproliferative diseases.

In phagocytic cells which are deprived of growth factors such as M-CSF, cell-cycle arrest occurs, whilst prolonged starvation leads to cell death through apoptosis (Li and Stanley, 1991). If growth factors are re-introduced following short periods of deprivation (12-18 hrs) re-entry into the cell-cycle occurs (Li and Stanley, 1991). There is a continual requirement for M-CSF from initiation of immediate early gene expression through G_1, therefore it is believed that M-CSF acts as a progression factor as well as a competence factor (Sherr, 1993). In the BAC1.2F5 cell line removal of M-CSF from cell culture results in quiescence and cell cycle arrest in G_1 (Morgan et al., 1987).

Serum starvation or incubation with PI 3-kinase inhibitors wortmannin and LY294002 induces apoptosis in PC12 cells and REF52 cells but not in NIH 3T3 or Balb 3T3 cells (Yao and Cooper, 1996; Yao and Cooper, 1995). Protection against apoptosis is conferred by PKB activation in Rat-1 cells and apoptosis is accelerated by PI 3-kinase inhibitors wortmannin and LY294002 (Kennedy et al., 1997). Additionally, neuronal cells transfected with wild-type PDGFr enter into apoptosis in the absence of PDGF or when the receptor possesses a mutated PI 3-kinase binding site (Carpenter and

Cantley, 1996). The role of PI 3-kinase in the prevention of apoptosis in macrophages and in other cell lineages has still to be fully defined.

Potent inhibitors of enzyme activity are extremely useful for determining what function the protein has in cells. Two specific PI 3-kinase inhibitors wortmannin and LY294002, have been used to characterise PI 3-kinase involvement in cell signalling. Wortmannin, produced by *Penicillium wortmannii*, specifically inhibits PI 3-kinase activity *in vitro* with an IC_{50} of 3nM (Arcaro and Wymann, 1993). At concentrations at or near IC_{50} wortmannin has no inhibitory effect on mammalian PI 4-kinase or S. *pombe* vps34p (Vlahos et al., 1994; Wymann et al., 1996). Once bound to PI 3-kinase, wortmannin blocks both the lipid and protein kinase functions of PI 3-kinase (Arcaro and Wymann, 1993; Barker et al., 1995; Martys et al., 1996). The Lilly Laboratories compound, LY294002, is a derivative of quercetin and has been shown to inhibit PI 3-kinase activity, *in vitro*, with an IC_{50} of 1.4μM (Vlahos et al., 1994). Although LY294002 is not as potent as wortmannin, it has the advantages that it is structurally unrelated to wortmannin and inhibits PI 3-kinase by a different mechanism, through the ATP-binding site on p110. These two inhibitors of PI 3-kinase will allow the characterisation of PI 3-kinase activity during M-CSF stimulated cell responses in BAC1.2F5 cells, including apoptosis and proliferation.

The main aims of chapter 5 were to determine whether PI 3-kinase activity was required for M-CSF stimulated proliferation in BAC1.2F5 macrophages. In addition the effect of withdrawal of serum and or M-CSF was also investigated. After determination that BAC1.2F5 cells require M-CSF and a factor in serum for protection against apoptosis the role of PI 3-kinase in M-CSF stimulated survival was also investigated with the PI 3-kinase inhibitors wortmannin and LY294002.

Although both LY294002 and wortmannin are specific inhibitors of PI 3-kinase, they possess different properties that can be exploited. Wortmannin is a more potent inhibitor of PI 3-kinase activity ($IC_{50} \approx$ 2-3nM) than LY294002 ($IC_{50} \approx 1.4\mu M$), but is inherently unstable with a $t_{½}$ of <30 minutes whereas LY294002 has a $t_{½}$ of >1 day. Therefore LY294002 was used in preference for experiments lasting longer than a day, and wortmannin was used for experiments lasting <3 hours.

To simplify interpretation I first present data on the effect of M-CSF and serum starvation on cell numbers before presenting data on the effects of withdrawal of these factors or inhibition of PI 3-kinase activity on BAC1.2F5 apoptosis.

5.1.0. BAC1.2F5 proliferation induced by M-CSF.

Stimulation of cell proliferation by M-CSF in BAC1.2F5 cells was characterised by the quantification of 5-bromo-2-deoxyuridine (BrdU) incorporation (chapter 3.3.7.) into *de novo* synthesised DNA in place of thymidine (Boehringer Mannheim). BrdU incorporation was detected via immunofluorescence and BrdU-positive cells were counted and expressed as a percentage of the total cell population. A dose response experiment showed that BAC1.2F5 cells proliferated in response to recombinant murine M-CSF (R&D systems) at concentrations as low as 1ng ml^{-1} (Fig. 5.1.1.).

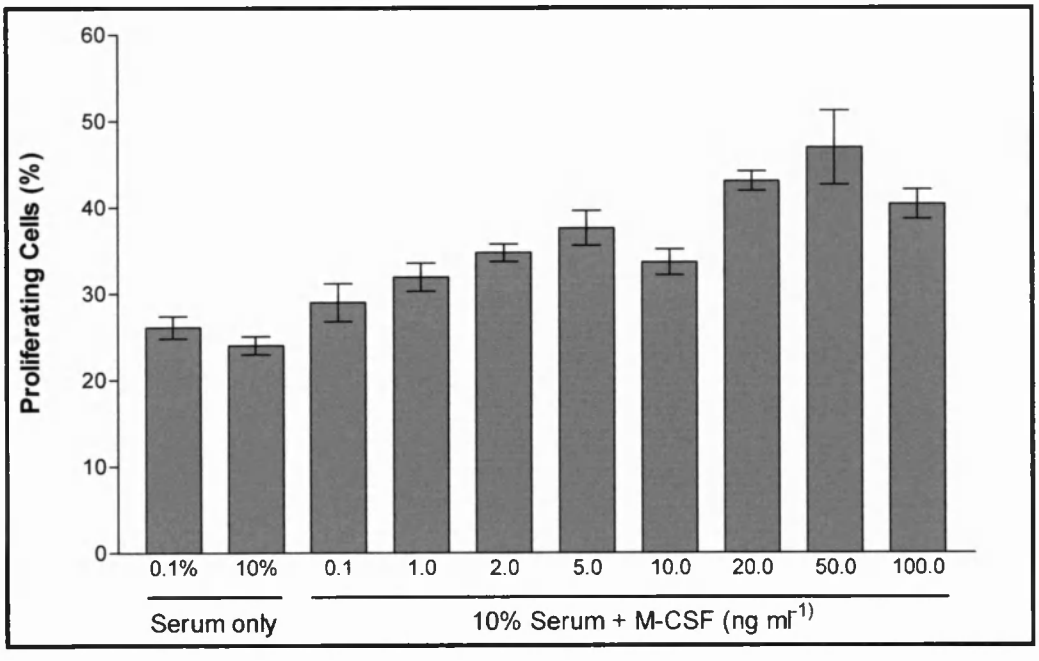


Fig. 5.1.1. The effect of recombinant murine M-CSF on the proliferation of BAC1.2F5 cells. BAC1.2F5 cells were seeded at a density of $1.5x10^4$ cells per 13mm coverslip, then cultured for 24 hours in DMEM/10%FCS (Gibco-BRL) without M-CSF. Cells were then exposed to varying concentrations (0.1-100ng ml^{-1}) of rmM-CSF (R&D systems) in fresh medium containing 10% foetal calf serum, cultured for a further 24 hours. Cells were labelled with BrdU and analysed by fluorescence microscopy. Error bars are ±S.E.M (*n=3*).

The proliferative response to M-CSF was dose dependent, however addition of M-CSF at 50.0ng ml^{-1} induced maximum proliferation over a 24 hour period. All further experiments used M-CSF at 50-100 ng ml^{-1}.

5.2.0. Requirement for PI 3-kinase during M-CSF stimulated proliferation in BAC1.2F5 cells

To determine whether PI 3-kinase was required for the observed increase in cell proliferation BAC1.2F5 cells that had been starved of M-CSF for 24 hours to induce quiescence were cultured for a further 24 hours in the presence of 50ng ml^{-1} M-CSF. During the M-CSF incubation, BAC1.2F5 cells were continually incubated with wortmannin at either 3 or 30nM and LY294002 at either 1.4μM or 10μM. Fig. 5.2.1. is a representative example of a BrdU stain, de novo synthesised DNA was stained green in cell nuclei. M-CSF stimulated a two fold increase in the numbers of proliferating cells compared to serum only controls (Fig. 5.2.2.). The PI 3-kinase inhibitors wortmannin and LY294002, at concentrations similar to their *in vitro* IC_{50} values or ten fold higher had no significant effect on the M-CSF stimulated increase in proliferating cells. This suggests that the PI 3-kinase activity which has previously been reported to be activated downstream of c-*fms* is not necessary for the proliferative signal mediated by c-*fms* in BAC1.2F5 macrophages.

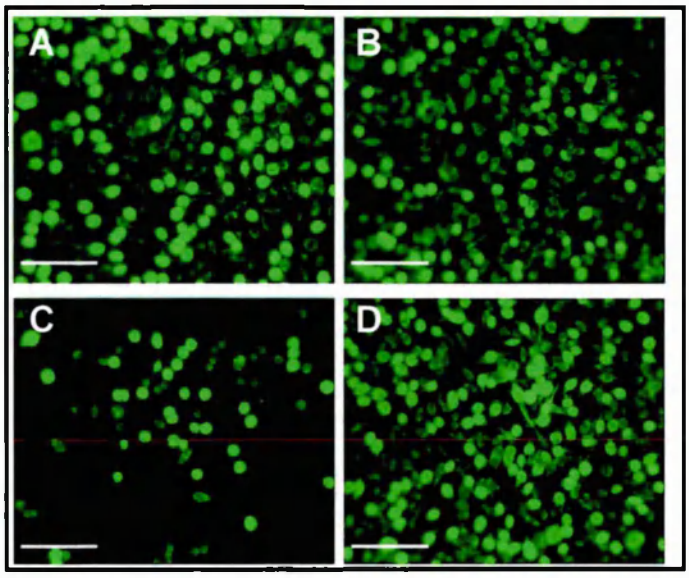


Fig. 5.2.1. The effect of PI 3-kinase inhibitors on M-CSF stimulated proliferation in BAC1.2F5 cells. BAC1.2F5 were seeded at a density of $1.5x10^4$ cells per 13mm coverslip, then cultured for 24 hours in DMEM/10%FCS without M-CSF. Cells were then incubated in fresh medium containing 10% foetal calf serum and cultured for a further 24 hours containing either 50ng ml^{-1} M-CSF (A), no M-CSF (B), 50ng ml^{-1} M-CSF and 30nM wortmannin (C) or 50ng ml^{-1} M-CSF and 10μM LY294002 (D). Cells were labelled with BrdU and photographed by fluorescence microscopy. Bars indicate 25μm.

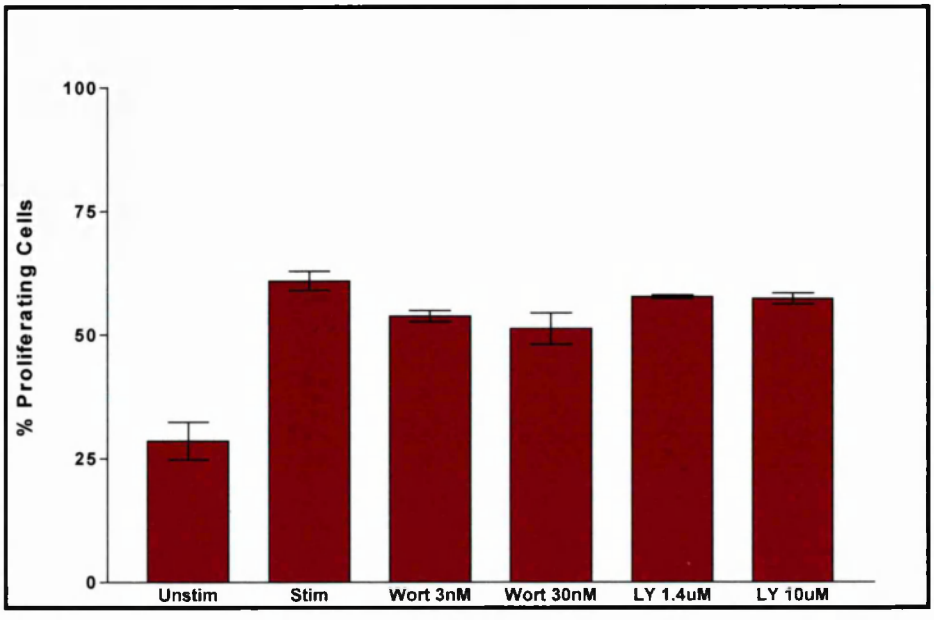


Fig. 5.2.2. Quantification of the effect of PI 3-kinase inhibitors on M-CSF stimulated proliferation in BAC1.2F5 cells. BAC1.2F5 cells were seeded at a density of $1.5x10^4$ cells per 13mm coverslip, and then cultured for 24 hours in DMEM/10%FCS without M-CSF. Cells were then incubated in fresh medium containing 10% foetal calf serum and cultured for a further 24 hours containing either no M-CSF, 50ng ml^{-1} M-CSF or 50ng ml^{-1} M-CSF and either 3nM wortmannin, 30nM wortmannin, 1.4μM LY294002 or 10μM LY294002. Cells were labelled with BrdU and analysed by fluorescence microscopy. Error bars are ±S.E.M (*n=3*).

5.3.0. Effect of M-CSF and/or serum withdrawal on BAC1.2F5 cell density

Routinely, BAC1.2F5 cells are cultured in medium supplimented with 10% serum and M-CSF therefore withdrawal of M-CSF, serum or both was investigated. Over a period of four days the increase in cell population was monitored by counting cells within a defined area. BAC1.2F5 cells cultured in the presence of 10% serum and M-CSF increase exponentially in cell density over time with an 8-fold increase by day four (Fig. 5.3.1., panel A).

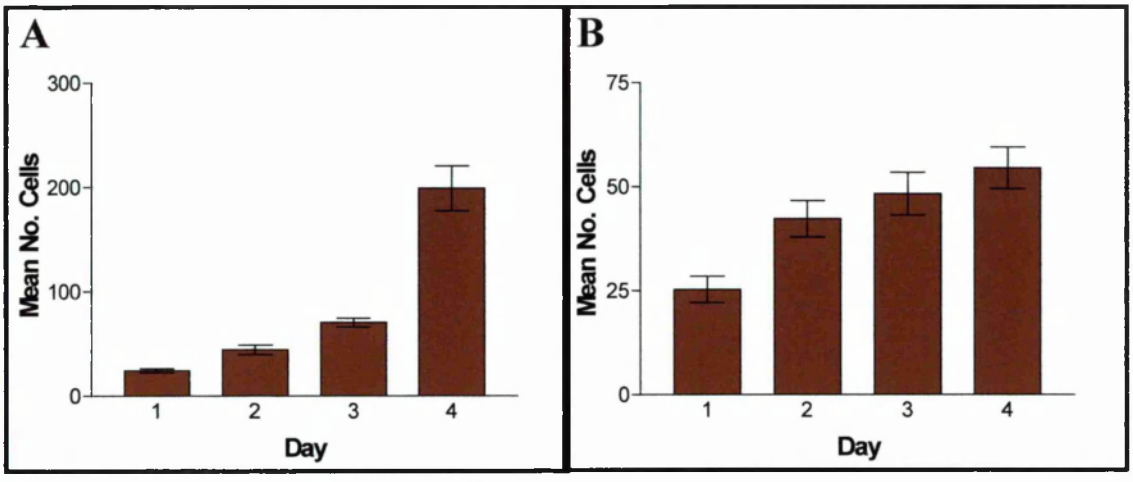


Fig. 5.3.1. Effect of M-CSF on BAC1.2F5 cell density in complete medium. BAC1.2F5 cells were seeded at a density of $1.5x10^4$ cells per 13mm diameter coverslip, then cultured in DMEM/10%FCS containing 50ng ml^{-1} M-CSF (A) or without M-CSF (B). Culture medium was replaced every 24 hours. Cells were analysed by light microscopy. Error bars are ±S.E.M (*n=3*).

BAC1.2F5 cells approximately double their cell density every 24 hours in the presence of M-CSF. However, withdrawal of M-CSF from the culture medium resulted cell a quite dramatic reduction in cell doubling to once every four days, compared to cells cultured with M-CSF (Fig. 5.3.1., panel B).

The role of serum factors was investigated by reducing the serum concentration to 0.1% (Fig. 5.3.2.). Under low serum conditions and without M-CSF the cell density does not increase in number but actually decreases over time (Fig. 5.3.2., panel A).

This is apparent by day 3, and results in a 40% loss in cell numbers by day four (Fig. 5.3.2., panel A). Addition of M-CSF does not rescue BAC1.2F5 cells from this reduction in cell numbers which suggests that a factor or factors present in serum are also required for BAC1.2F5 survival in culture (Fig. 5.3.2., panel B). This survival factor does not appear to be M-CSF.

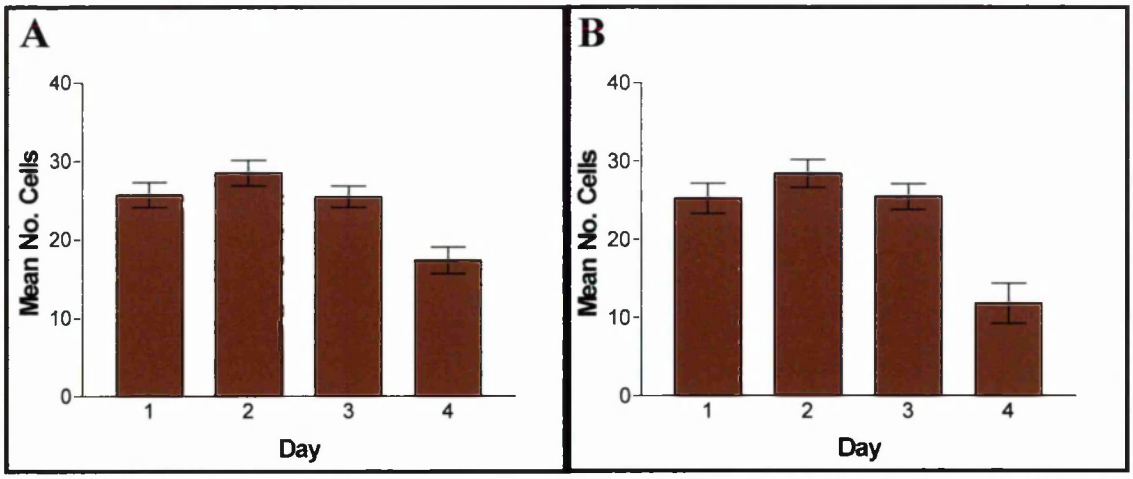


Fig. 5.3.2. Effect of low serum on BAC1.2F5 cell density. BAC1.2F5 cells were seeded at a density of $1.5x10^4$ cells per 13mm diameter coverslip, then cultured in DMEM/0.1%FCS containing 50ng ml^{-1} M-CSF (A) or without M-CSF (B). Culture medium was replaced every 24 hours. Cells were analysed by light microscopy. Error bars are ±S.E.M (*n=3*).

5.4.0. Effect of delipidated serum and LPA on BAC1.2F5 cell density

Recently, Koh *et al* have identified a noncytokine component of serum that protects peritoneal macrophages from serum starvation-induced apoptosis (Koh et al., 1998). This constituent of serum is lysophosphatidic acid, (LPA), a glycerophospholipid normally found in serum (Koh et al., 1998). LPA induced macrophage survival was reportedly blocked by wortmannin or LY294002 (Koh et al., 1998). To determine whether LPA was the serum survival factor for BAC1.2F5 cells, the effect of recombinant LPA or lipid-depleted serum on BAC1.2F5 macrophage survival was investigated (Fig.5.4.1.).

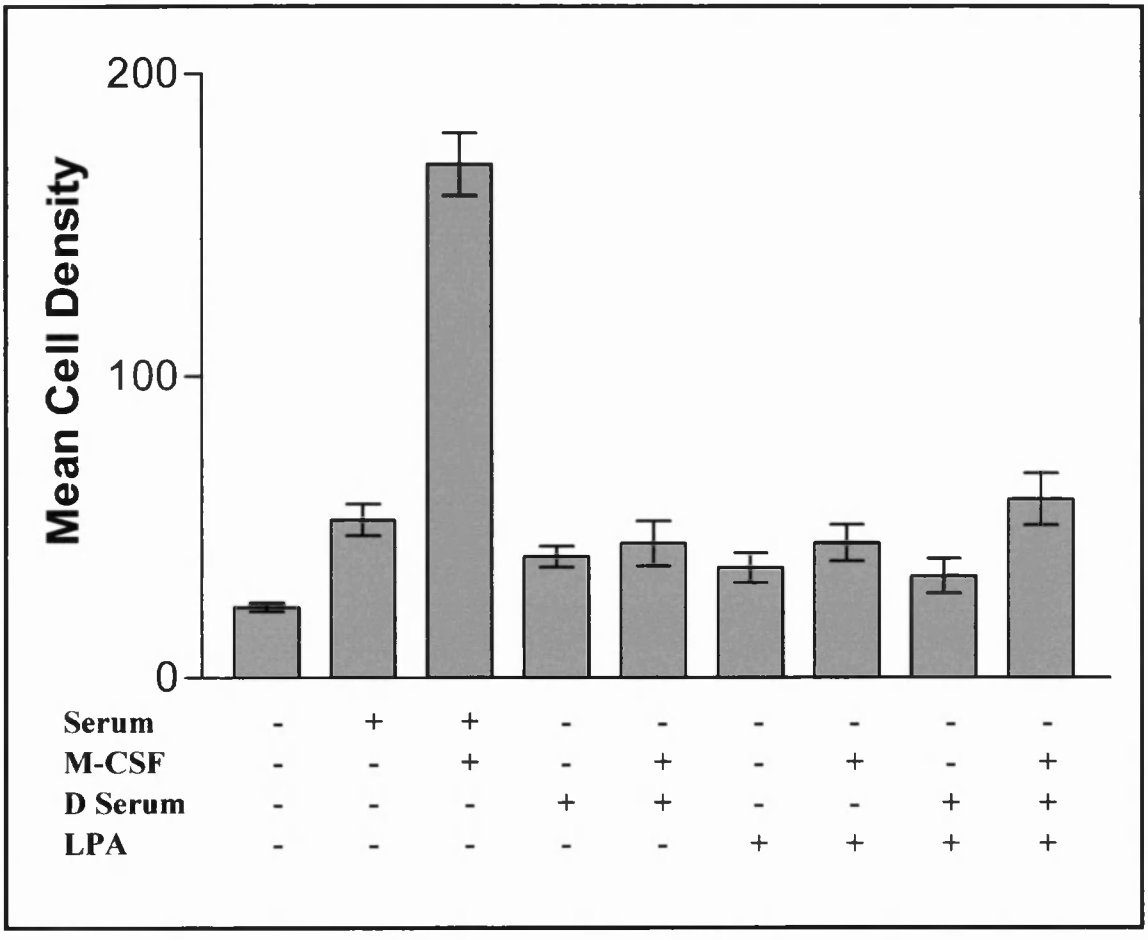


Fig. 5.4.1. Effects of serum, M-CSF and LPA withdrawal on BAC1.2F5 cell density after 4 days. BAC1.2F5 cells were cultured as above for 4 days. Culture medium was replaced every 24 hours. Cells were analysed by light microscopy. Error bars are ±S.E.M (*n=3*).

In the absence of serum and M-CSF there was no increase in cell numbers. However, incubation with serum doubled the cell density and incubation with serum and 50ng ml^{-1} M-CSF stimulated an eight fold increase in cell density (Fig.5.4.1.). Supplementing cells with delipidated serum alone increased cell density compared to no serum control but not to the same extent as in the presence of 10% serum (Fig.5.4.1.). Adding 50ng ml^{-1} M-CSF to the delipidated serum did not affect the cell density, which suggests that a lipid component of serum may be required for cell proliferation (Fig.5.4.1.). Serum has been estimated to contain 100μM LPA, therefore routine cell culture with 10% serum contains 10μM LPA. Cells cultured in the absence of normal serum were also supplemented with 10μM LPA, however this had

no effect cell density except for delipidated serum containing M-CSF where a partial increase in cell density occurred (Fig.5.4.1.). These data suggest that a lipid component of serum is required for full stimulation of cell proliferation by M-CSF and can be partially restored by 10μM LPA. However LPA alone did not increase cell numbers and neither could LPA substitute for 10% serum in combination with M-CSF. However LPA, delipidated serum and M-CSF stimulated a small increase in cell numbers compared to cells cultured in the absence of delipidated serum. However this effect did not represent full reconstitution of the effects of 10% serum and M-CSF normally observed. These data suggest that LPA is one co-factor necessary for the full M-CSF-stimulated proliferative response in BAC1.2F5 cells, but there is at least one other factor that is absent after delipidation of serum.

5.5.0. Effect of the PI 3-kinase inhibitor LY294002 on M-CSF and serum stimulated BAC1.2F5 cell survival

Although M-CSF stimulated proliferation of BAC1.2F5 cells does not appear to require PI 3-kinase activity, PI 3-kinase activity may still be required for survival in culture. To investigate whether PI 3-kinase activity was required for BAC1.2F5 survival the experiments in section 5.3.0. were repeated but in the presence of LY294002 at 10μM. Addition of LY294002 to BAC1.2F5 cells cultured in 0.1% serum without M-CSF accelerated the rate of cell death measured by gross cell counts (Fig. 5.5.1., panel A). On day four less than 25% of the seeded population was left and the effect of LY294002 was apparent as early as day 2 (Fig. 5.5.1., panel A). The effect of LY2940002 on cell density was not rescued by supplementing the culture medium with serum alone (Fig. 5.5.1., panel B), suggesting that PI 3-kinase activity is required for the serum factor induced survival of BAC1.2F5 macrophages.

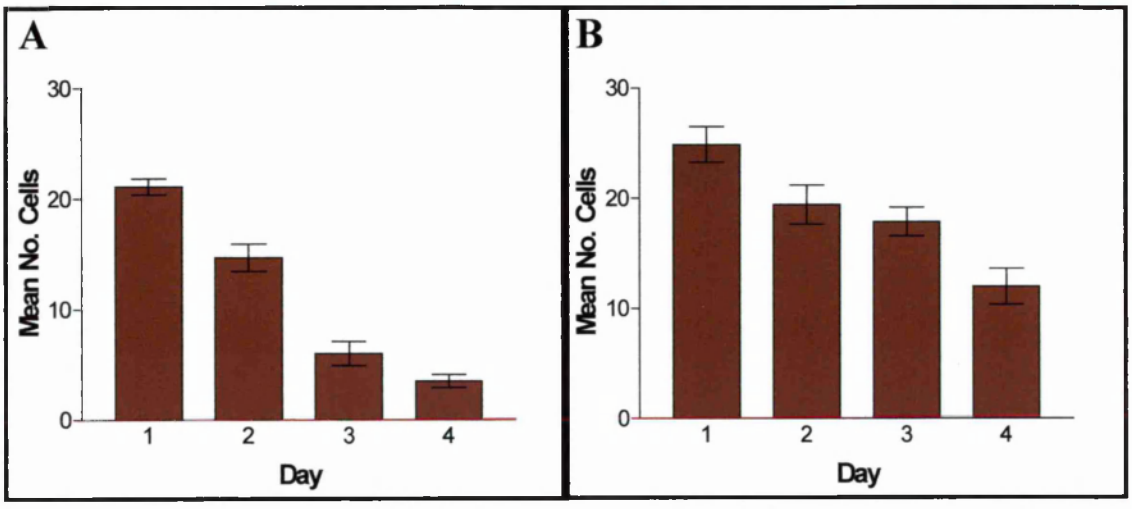


Fig. 5.5.1. Effect of LY294002 on cell density of BAC1.2F5 cells cultured in low serum without M-CSF. BAC1.2F5 cells were seeded at a density of $1.5x10^4$ cells per 13mm diameter coverslip, then cultured in 0.1%FCS (A) or 10%FCS (B) containing 10μM LY294002 without M-CSF. Culture medium was replaced every 24 hours. Cells were analysed by light microscopy. Error bars are ±S.E.M (*n=3*).

In addition the effect of LY2940002 on cell density was not rescued by supplementing the culture medium with M-CSF alone (Fig. 5.5.2., panel A), again suggesting that PI 3-kinase activity is required during M-CSF induced survival in BAC1.2F5 macrophages.

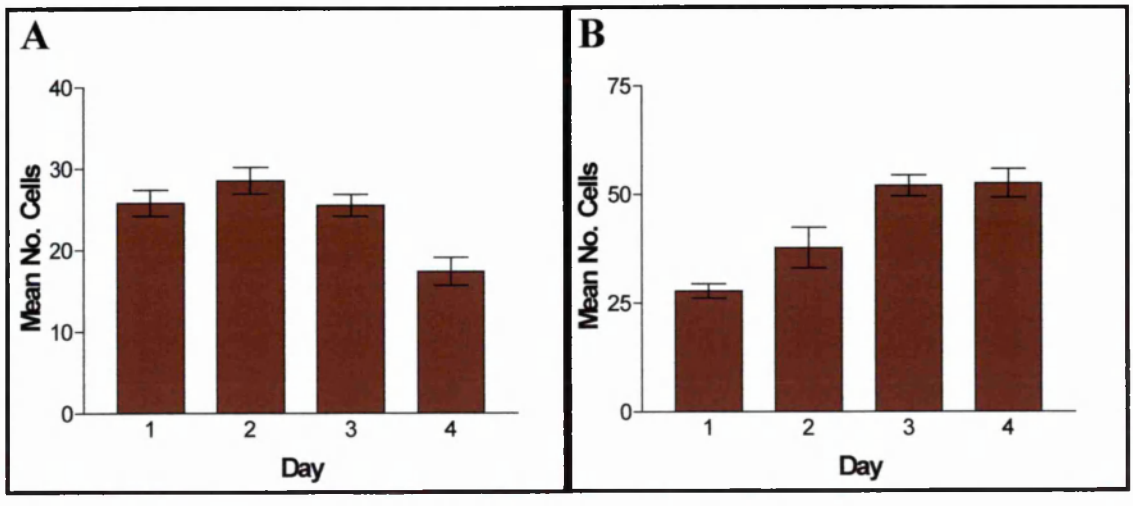


Fig. 5.5.2. Effect of LY294002 on BAC1.2F5 cell density in medium containing 0.1% or 10% FCS supplemented with 50ng ml^{-1} M-CSF. BAC1.2F5 cells were seeded at a density of $1.5x10^4$ cells per 13mm diameter coverslip, then cultured in 0.1% FCS (A) or 10% FCS (B) containing 10μM LY294002 and 50ng ml^{-1} M-CSF. Culture medium was replaced every 24 hours and cells were analysed by light microscopy. Error bars are ±S.E.M (*n=3*).

When LY294002 was added to BAC1.2F5 cells cultured with 10% serum and 50ng ml^{-1} M-CSF, there was no cell death and a small increase in cell numbers (Fig. 5.5.2., panel B). However, the increase in cell numbers was not comparable to that seen in controls in the absence of LY 294002 (Fig. 5.3.1., panel A).

Fig. 5.5.3. compares the data from Fig. 5.3.1., panel A and Fig. 5.5.2, panel B. It is apparent from Fig. 5.5.3. that addition of 10μM LY294002 significantly inhibited M-CSF stimulated increases in BAC1.2F5 cell density ($P<0.0001$).

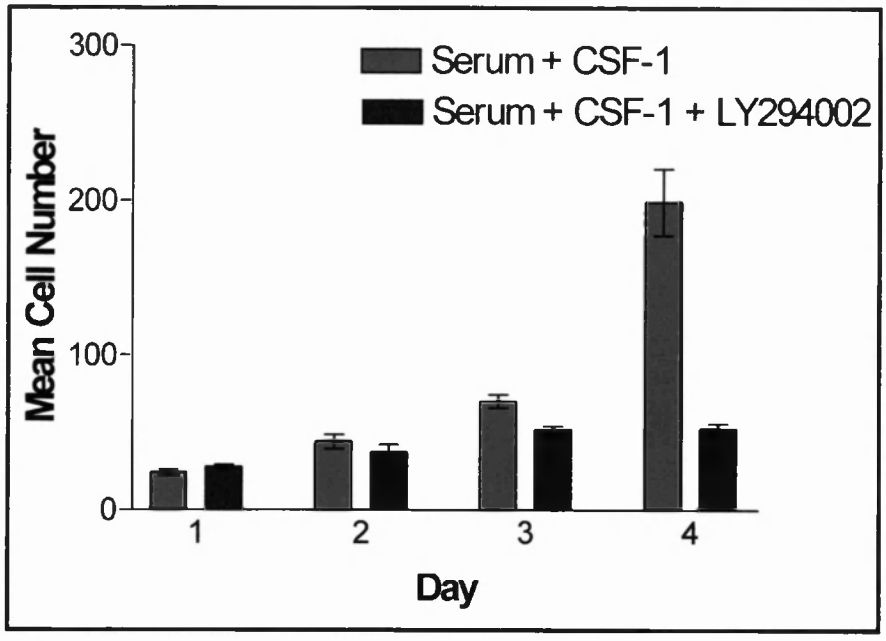


Fig. 5.5.5. Effects of LY294002 on BAC1.2F5 cell numbers when cultured in the presence of 10% FCS and 50ng ml^{-1} M-CSF. This figure summarises data from Figs. 5.3.1., panel A and 5.5.2., panel B. $P<0.0001$. Error bars are ±S.E.M ($n=3$).

5.6.0. Apoptosis induced by M-CSF and/or serum withdrawal in BAC1.2F5 cells

One of the early events during apoptosis in most cell types is the translocation of phosphatidylserine from the inner surface of the plasma membrane to the cell surface (Martin *et al* 1995). Phosphatidylserine can then be detected in living cells with a FITC conjugate of annexin V. In addition, the latter stages of apoptosis are characterised by living cells becoming necrotic, allowing the uptake of propidium iodide into DNA. This only occurs during once the plasma membrane becomes permeable during the late stages of apoptosis. The effects of serum and M-CSF withdrawal from BAC1.2F5 cultures were characterised either by annexin V or propidium iodide staining (chapter 3.3.8.). Comparative experiments showed that there was little difference in the data obtained between annexin V staining and PI staining, although PI staining appears shortly after annexin V staining (Fig. 5.6.1.).

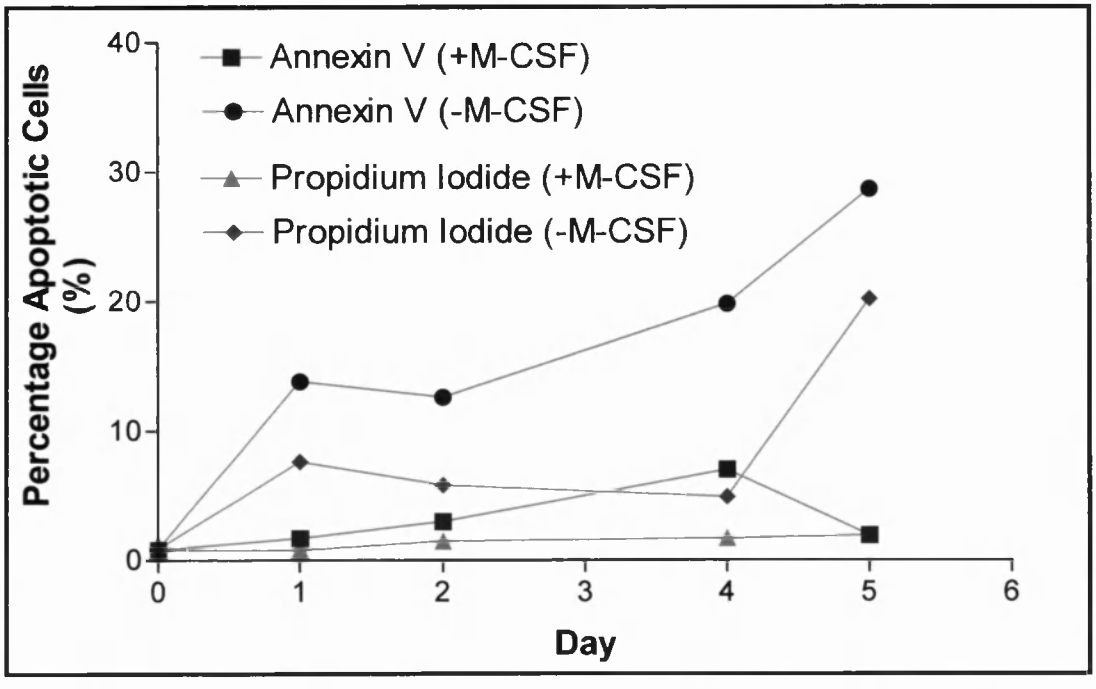


Fig. 5.6.1. Effect of M-CSF withdrawal on BAC1.2F5 apoptosis measured by Annexin V and Propidium Iodide staining. BAC1.2F5 cells were cultured for five days in the presence or absence of 50ng ml^{-1} M-CSF. Culture medium was replaced every 24 hours. Cells were stained with either an Annexin V-FITC conjugate or with propidium iodide (Clontech). Positive cells were documented by fluorescence photomicroscopy.

PI staining was consistently stronger than annexin V, and more easily detected by fluorescence microscopy, and therefore PI staining was used as a probe for apoptotic cells in the following series of experiments. Apoptosis induced by M-CSF withdrawal was detected after day 4 and was characterised by a 4-fold increase in the numbers of apoptotic cells detected (Fig. 5.6.1.).

To establish whether PI 3-kinase activity was required for M-CSF mediated BAC1.2F5 survival, replicate experiments to chapter 5.5.0. were performed except cells were stained with propidium iodide to identify apoptotic cells. Fig. 5.6.2. shows the percentage of cells undergoing apoptosis in BAC1.2F5 cells cultured in medium containing 10% FCS with or without 50ng ml^{-1} M-CSF. The percentage apoptosis was no more than 4% over the 4 days (Fig. 5.6.2., panel A).

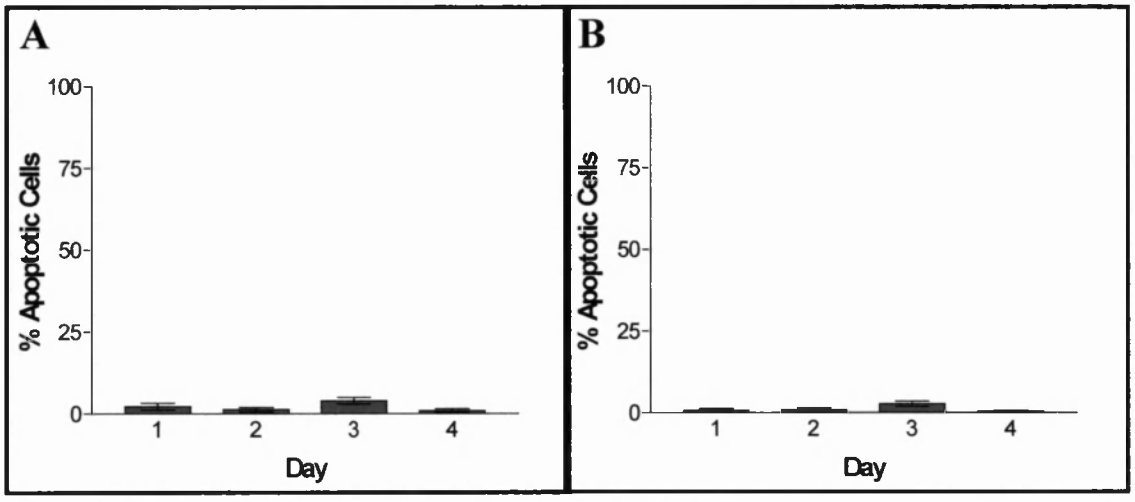


Fig. 5.6.2. Effect of M-CSF on BAC1.2F5 apoptosis measured by propidium iodide staining. BAC1.2F5 cells were cultured for four days in the presence (A) or absence (B) of 50ng ml^{-1} M-CSF. Culture medium was replaced every 24 hours. Cells were stained for 15 minutes with propidium iodide (Clontech). Positive cells were documented by fluorescence photomicroscopy. Error bars are ±S.E.M (*n=3*).

Withdrawal of M-CSF from the culture medium should not have had a measurable effect by day 4 based on the observations in Fig. 5.6.1. and indeed there was no

appreciable increase in detectable apoptosis in BAC1.2F5 cells cultured without M-CSF over 4 days (Fig. 5.6.2., panel B).

Cells were then cultured in 0.1% serum with and without 50 ng ml^{-1} M-CSF to determine whether M-CSF alone can confer protection against apoptosis in BAC1.2F5 cells (Fig. 5.6.3.). Removal of serum from the culture medium did not dramatically increase the detection of apoptotic cells although by days 3 and 4 there was an increase in apoptosis to a maximum of 9% (Fig. 5.6.3., panel A). Withdrawal of both serum and M-CSF apoptosis in BAC1.2F5 cells, but this was only detected on day 3 (20%) and day 4 (30%) (Fig. 5.6.3., panel B).

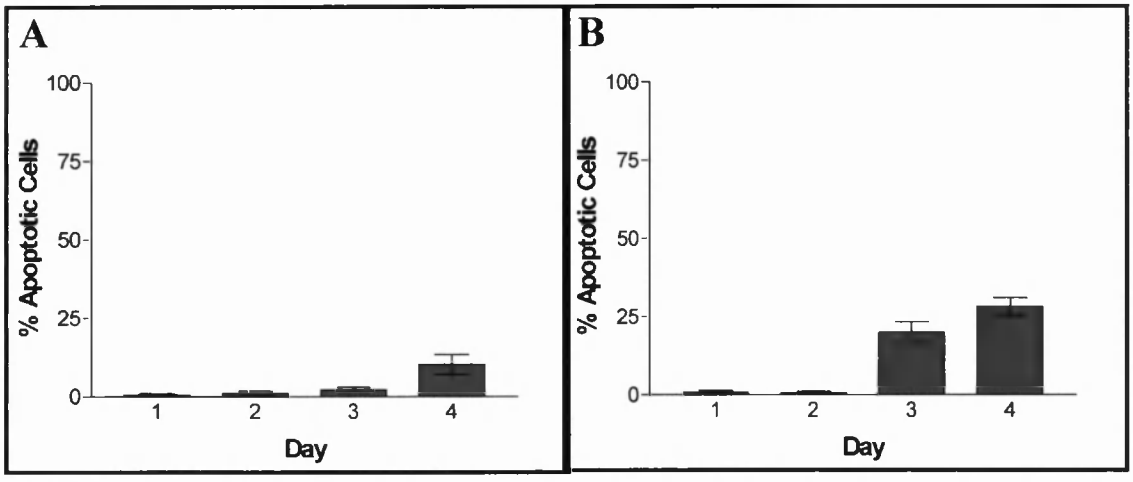


Fig. 5.6.4. Effect of serum withdrawal on BAC1.2F5 apoptosis measured by propidium iodide staining. BAC1.2F5 cells were cultured for four days in 0.1% serum in the presence (A) or absence (B) of 50ng ml^{-1} M-CSF. Culture medium was replaced every 24 hours. Cells were stained with propidium iodide for 15 minutes (Clontech). Positive cells were documented by fluorescence photomicroscopy. Error bars are ±S.E.M (*n=3*).

5.7.0. Apoptosis induced by LY294002 in BAC1.2F5 cells

Removal of either serum or M-CSF from culture medium induced apoptosis in a proportion of BAC1.2F5 cells. To determine if PI 3-kinase activity was involved in protection against apoptosis the PI 3-kinase inhibitor LY 294002 was incubated with BAC1.2F5 cells in the presence or absence of either serum or M-CSF or both (Figs.

5.7.1. and 5.7.2). Incubation of LY294002 with BAC1.2F5 cells in the absence of serum and M-CSF induced significant levels of apoptosis (Fig. 5.7.1., panel A). Apoptosis was apparent by day 3 with almost 60% of the cell population being apoptotic, increasing to 70% by day 4 (Fig. 5.7.1., panel A).

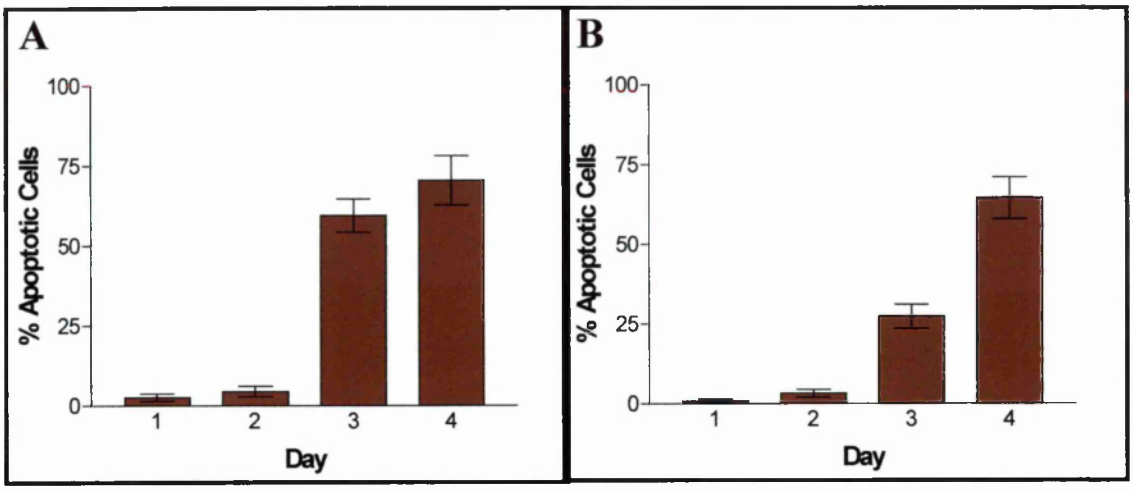


Fig. 5.7.1. Effect of LY294002 on apoptosis in BAC1.2F5 cells cultured in 0.1% serum without M-CSF measured by propidium iodide staining. BAC1.2F5 cells were cultured for four days in 0.1% serum, containing 10μM LY294002 in the absence (A) or presence (B) of 50ng ml^{-1} M-CSF. Culture medium was replaced every 24 hours. Cells were stained with propidium iodide for 15 minutes (Clontech). Positive cells were documented by fluorescence photomicroscopy. Error bars are ±S.E.M (*n=3*).

Addition of M-CSF to BAC1.2F5 cells cultured in 0.1% serum and 10μM LY 294002 reduced the LY294002 induced increase in apoptosis at day 3 to 30%, half the percentage observed in the absence of M-CSF (Fig. 5.7.1., panel B). However by day 4 the percentage of apoptotic cells was almost 70%, similar to that observed on day 4 in the absence of M-CSF (Fig. 5.7.1., panel B).

Addition of 10% serum to BAC1.2F5 cells cultured in 10μM LY 294002 reduced the LY294002 induced increase in apoptosis at day 3 to 15%, a quarter of the percentage observed in the absence of M-CSF (Fig. 5.7.2., panel A). By day 4 the percentage of

apoptotic cells was almost 40%, not as high as that observed on day 4 in the absence of M-CSF (Fig. 5.7. 2., panel A). Culture of BAC1.2F5 cells in the presence of both serum and M-CSF significantly reduced the increase in apoptosis detected by day 4 induced by 10μM LY294002 (Fig. 5.7. 2., panel B).

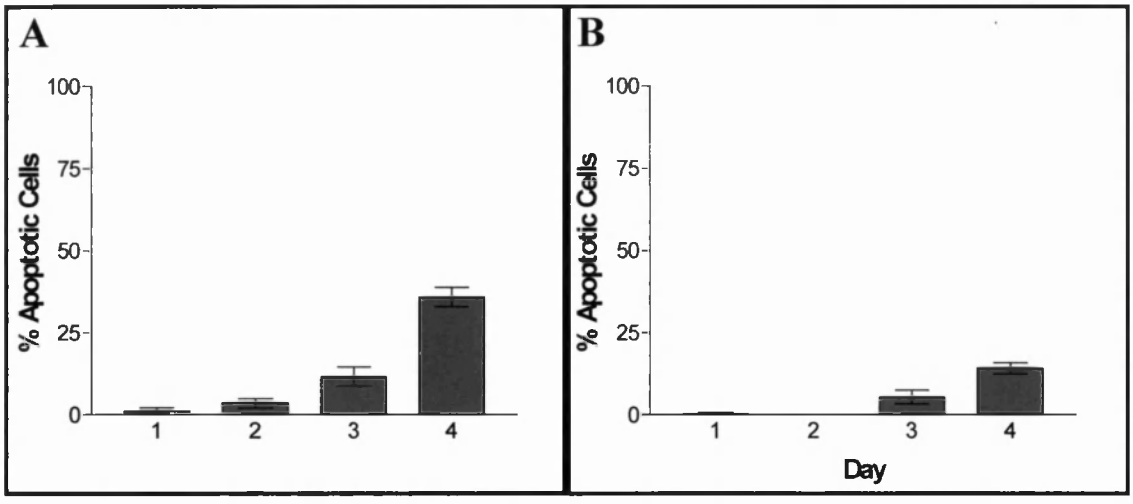


Fig. 5.7.2. Effect of LY294002 on apoptosis in BAC1.2F5 cells cultured in 10% serum and 50ng ml^{-1} M-CSF measured by propidium iodide staining. BAC1.2F5 cells were cultured for four days in 10% serum, containing 10μM LY294002 in the absence or presence of 50ng ml^{-1} M-CSF. Culture medium was replaced every 24 hours. Cells were stained with propidium iodide for 15 minutes (Clontech). Positive cells were documented by fluorescence photomicroscopy. Error bars are ±S.E.M (*n=3*).

Direct comparison of BAC1.2F5 cells cultured in 10% serum and 50ng ml^{-1} M-CSF to cells cultured identically but including 10μM LY 294002 demonstrates that, although in relation to the total cell population analysed there are only very subtle changes observed, there was a significant (P<0.0001) difference (Fig. 5.7.3.). Addition of LY 294002 to normal BAC1.2F5 cultured cells induced a greater than 10 fold increase in the number of apoptotic cells by day 4 (Fig. 5.7.3.).

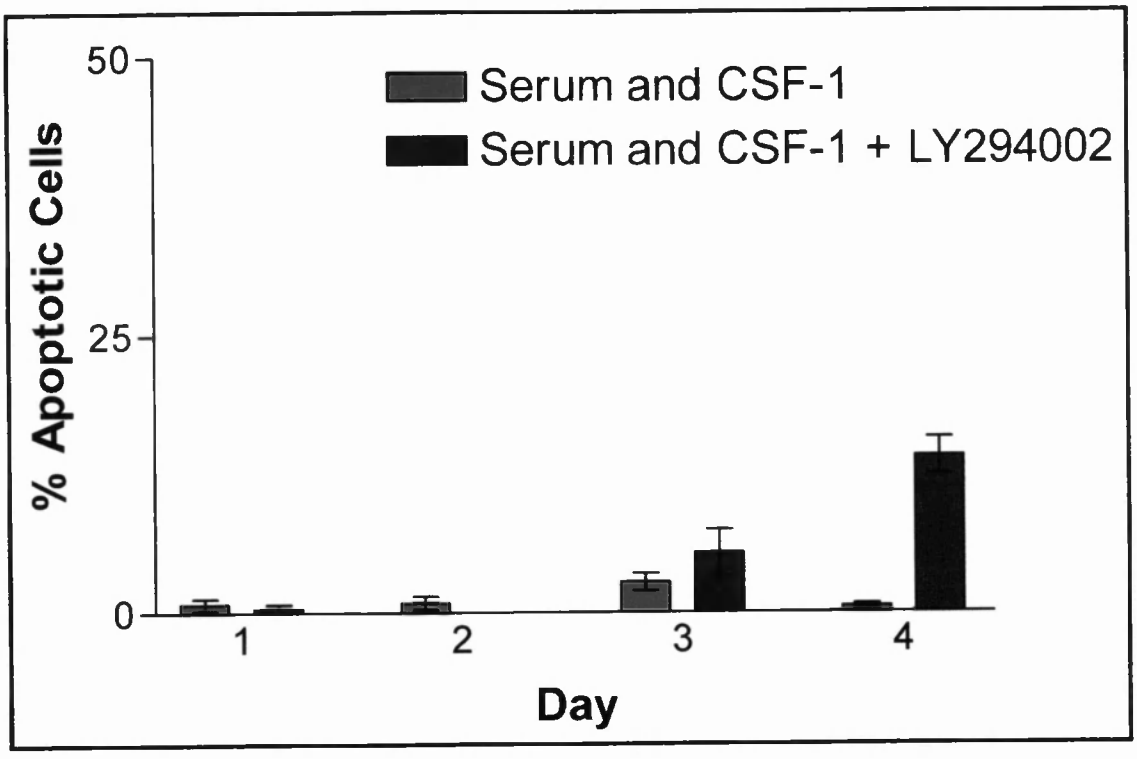


Fig. 5.7.3. Effects of LY294002 on BAC1.2F5 cell survival measured by detection of apoptotic cells. This figure is a comparison of the data represented in Figs. 5.6.2., panel A and 5.7.2., panel B. $P<0.0001$. Error bars are ±S.E.M ($n=3$).

5.8.0. Effect of delipidated serum and LPA on BAC1.2F5 apoptosis

The effect of LPA on BAC1.2F5 macrophage apoptosis, measured by propidium iodide uptake, was investigated by incubating cells with lipid depleted serum over 4 days in the presence or absence of M-CSF or LPA (Fig. 5.8.1.). In the absence of serum and M-CSF BAC1.2F5 macrophages become apoptotic with almost 55% of cells present staining positive (Fig. 5.8.1.). Addition of 10% serum almost completely blocked apoptosis and this was augmented by incubation with 50ng ml^{-1} M-CSF (Fig. 5.8.1.).

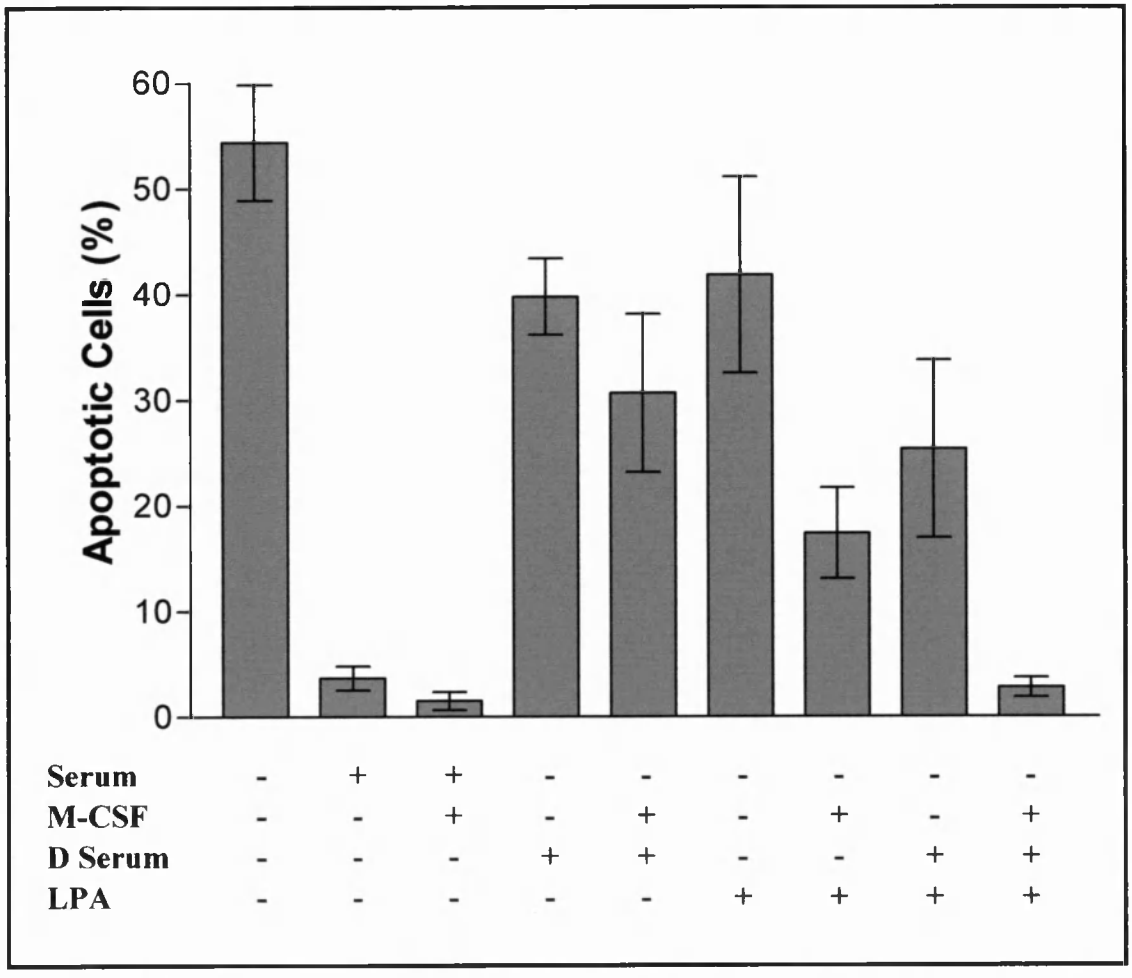


Fig. 5.8.1. Effects of sreum, M-CSF and LPA withdrawal on BAC1.2F5 apoptosis after 4 days. BAC1.2F5 cells were cultured as above for 4 days. Culture medium was replaced every 24 hours. Cells were analysed by fluorescence microscopy. Error bars are ±S.E.M (*n=3*).

Cells cultured in delipidated serum were apoptotic (≈ 40%) but this was lower than in the complete absence of serum and was reduced further, but not rescued completely

with M-CSF. This suggests that a lipid factor found in serum provides a limited protection against apoptosis in BAC1.2F5 macrophages. Addition of 10μM LPA to cells cultured in the absence of serum or M-CSF did not prevent apoptosis (≈42%), however inclusion of either M-CSF or delipidated serum significantly reduced the number of apoptotic cells (≈18% and ≈25%, respectively) (Fig. 5.8.1.). Culture of BAC1.2F5 cells in delipidated serum supplemented with M-CSF and LPA reduced the levels of apoptosis to those seen in serum and M-CSF/serum controls (Fig. 5.8.1.). These data suggest that the lipid factor in serum that is required for protection against apoptosis in BAC1.2F5 macrophages may be LPA. However, LPA is not the only factor in serum required for BAC1.2F5 survival since delipidated serum also contains a component that prevents maximal induction of apoptosis. In addition, M-CSF and LPA act synergistically to inhibit apoptosis and this is further enhanced by a third factor or factors present in serum.

5.9.0. Discussion

BAC1.2F5 cells are a sub-clone of the BAC1 cell line and depend on M-CSF for survival and proliferation and have previously been shown to respond in a dose dependent manner to increasing concentrations of recombinant murine M-CSF growth factor (Morgan et al., 1987). This was confirmed for the BAC1.2F5 clone obtained from E. Richard Stanley (AECOM, New York) (Fig. 5.1.1.). Treatment of BAC1.2F5 cells with M-CSF induced cell proliferation in a dose dependent manner with a maximal 2-fold increase in DNA synthesis after 24 hours stimulated by 50ng ml^{-1} M-CSF (Fig. 5.1.1.). All future experiments with BAC1.2F5 cells were carried out at either 50 or 100ng ml^{-1} M-CSF to stimulate maximum cell activation.

It has previously been demonstrated that loss of Tyr^{723} of c-*fms*, the key binding site for PI 3-kinase reduced M-CSF stimulated proliferation in transfected Rat-2 cells but did not block DNA synthesis stimulated by M-CSF in transfected NIH 3T3 cells (Shurtleff et al., 1990; van der Geer and Hunter, 1993). Apart from the different cell backgrounds the only differences in these studies were that in Rat-2 fibroblasts, both the PI 3-kinase and the Grb-2 binding site were mutated but only the PI 3-kinase binding site in NIH 3T3 cells. Therefore it is possible that although the major PI 3-kinase binding site is Tyr^{723}, PI 3-kinase activation can occur indirectly via Grb-2 since the Grb-2 SH2 domain has been shown to associate with activated PI 3-kinase in human monocytes (Saleem et al., 1995). It is also possible that only Grb2 binding is involved in proliferation and therefore loss of both binding sites would abrogate direct and indirect activation of PI 3-kinase. The possible involvement of PI 3-kinase in M-CSF stimulation of proliferation in macrophages was investigated in the BAC1.2F5 cell line using the PI 3-kinase-specific inhibitors wortmannin and LY294002 (Figs. 5.2.1. and 5.2.2.). DNA synthesis was quantified by BrdU incorporation and Fig.

5.2.1. illustrates a typical experiment. Addition of either wortmannin or LY294002 at concentrations which have previously been reported to inhibit PI 3-kinase activity *in vitro* had little or no effect on M-CSF-stimulated cell proliferation present 24 hours after M-CSF stimulation (Fig. 5.2.2.). Therefore it appears that initiation of de novo DNA synthesis and cell proliferation is not dependent on PI 3-kinase activation in BAC1.2F5s and probably macrophages in general.

BAC1.2F5 cells have been extensively characterised with respect to cell morphology and although M-CSF has been demonstrated to be required for BAC1.2F5 survival, the exact requirement for PI 3-kinase activity for M-CSF mediated survival has yet to be investigated. Before investigating the role of PI 3-kinase, the effects of M-CSF and serum withdrawal on BAC1.2F5 cell numbers were studied in order to characterise fully the normal BAC1.2F5 responses. Stimulation of quiescent BAC1.2F5 cells in the presence of 10% serum caused cell doubling every 24 hours resulting in an 8-fold increase in cell numbers observed by day four. The absence of M-CSF dramatically inhibited cell proliferation and only a 2-fold increase in cell numbers was observed by day 4 (Fig. 5.3.1.). In addition, the net effect on cells cultured in low serum, (0.1%) and in the absence of M-CSF was no net gain but a reduction of cell numbers by day 4 (Fig. 5.3.2.). Addition of M-CSF did not rescue this response (Fig. 5.3.2.). Therefore, although M-CSF is required for BAC1.2F5 proliferation, it is not the only factor required and must act synergistically with another factor, or factors present in foetal calf serum. Recently it has been noted that lysophosphatidic acid (LPA), a phospholipid constituent of serum is required by macrophages for protection against apoptosis mediated by a PI 3-kinase dependent pathway with similar potency to M-CSF (Koh et al., 1998). Incubation of BAC1.2F5 cells with delipidated serum, which does not contain LPA, but supplemented with

either synthetic LPA or M-CSF or both did not mimic the increases in cell numbers observed in the presence of normal serum and M-CSF (Fig. 5.4.1.). This suggests that although M-CSF stimulated the survival/proliferation of BAC1.2F5 macrophages in normal serum conditions, there is a serum factor which is removed by serum delipidation, that may also be required for BAC1.2F5 survival or proliferation.

Addition of the PI 3-kinase inhibitor, LY294002, induced cell death in low serum conditions resulting in substantial cell loss (Fig. 5.5.1., panel A). However, in the presence of 10% serum (Fig. 5.5.1., panel A), there was marginally less cell loss compared to low serum conditions (Fig. 5.5.1.). Addition of M-CSF to cells cultured in low serum had a similar effect on cell death as 10% serum, but did not prevent the PI 3-kinase inhibitor from blocking cell survival (Fig. 5.5.2.). The presence of LY294002 blocked the 8-fold increase in cell numbers observed in the presence of 10% serum and M-CSF in that there was only a 2-fold doubling in cell numbers (Fig. 5.5.2.). It appears that there is another factor required in addition to M-CSF for BAC1.2F5 survival or proliferation. What is appearing is a scenario where there is a balance between survival and proliferation in BAC1.2F5 cells which is not regulated by M-CSF alone. The increase in cell numbers of BAC1.2F5 cells after 2-3 days requires M-CSF and serum components which are both PI 3-kinase dependent.

The previous results showing an effect of PI 3-kinase inhibitors on M-CSF-induced increases in cell numbers, but little effect on proliferation, strongly suggested that a major effect of these inhibitors was on cell survival. To formally investigate the role of PI 3-kinase activity in cell survival the effect of LY294002 on apoptosis was investigated. It is known that PI 3-kinase activity is essential for the activation of a pathway, downstream of various growth factor receptors, that leads to a protection

against apoptosis in a number of cell types. PI 3-kinase is an integral part of pathways by which insulin and NGF prevent apoptosis (Minshall et al., 1996), and PI 3-kinase products bind to and activate PKB, with high affinity (Alessi et al., 1997). Activation of PKB in various cell lines has been shown to stimulate survival, proliferation, differentiation, GLUT4 translocation, glycogen synthase kinase-3 (GSK3) down-regulation and up-regulation of E2F transcription factor expression (Marte and Downward, 1997). Phospholipid vesicles containing $PI_{3,4}P_2$ specifically bind to PKB via its PH domain causing dimerisation and activation of PKB, *in vitro* (Franke et al., 1997; Klippel et al., 1997). Association of $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ with the PH domain of PKB cause its translocation to the plasma membrane enabling the PKB kinase, PDK-1, to phosphorylate and activate the PKB kinase activity (Stephens et al., 1998).

Measurement of cell survival or apoptosis was carried out either by annexin V or propidium iodide (PI) uptake. Annexin V is a cell surface marker of one of the early events during apoptosis whereas PI uptake into DNA only occurs in the latter stages of apoptosis, in necrotic cells. Either method is a valid marker for apoptosis and both were used to characterise apoptosis induced by M-CSF withdrawal from BAC1.2F5 cells (Fig. 5.6.1.). Annexin V staining indicated that apoptosis was stimulated as early as 24 hours after M-CSF withdrawal and increased over time, however staining was relatively weak. In contrast PI staining only indicated apoptosis after day 4, however PI staining was much stronger and so PI staining was selected in preference to annexin V as a marker for apoptosis in the latter experiments. Therefore in light of this data it is assumed that M-CSF is required for BAC1.2F5 survival. The experiments on BAC1.2F5 survival and apoptosis in the presence of M-CSF and high/low serum concentrations were replicated using the PI 3-kinase inhibitor wortmannin at 100nM, which gave essentially the same results (data not shown).

The effects of M-CSF, serum and LPA on apoptosis were investigated over a 4 day time period. As indicated there was little or no apoptosis in the presence of both 10% serum and M-CSF (Fig. 5.6.2., panel A), and M-CSF withdrawal had no effect (Fig. 5.6.2., panel B) thus 10% serum alone was sufficient for protection against apoptosis over this time period. Withdrawal of serum did have a small effect on apoptosis by day 4 (≈ 12%) and this was augmented by the withdrawal of M-CSF as well (Fig. 5.6.4., panel B). Under these conditions apoptosis was detected by day 3 and over 25% of cells were apoptotic by day 4. Addition of LY294002 substantially accelerated apoptosis in M-CSF and serum deprived BAC1.2F5 cells and apoptosis was detected by day 3 with almost 75% of cells being apoptotic by day 4 (Fig. 5.7.1., panel A). The effect of LY294002 was slightly reduced by addition of M-CSF to cells grown in low serum, but apoptosis was still evident and at least 70% of cells were apoptotic by day 4 (Fig. 5.7.1., panel B). Replacing M-CSF with serum had a more dramatic effect, and LY294002-induced apoptosis was reduced but not to levels observed in the absence of LY294002 (Figs. 5.7.2., panel A and 5.6.2., panel B). However this reduction in apoptosis was augmented by addition of M-CSF (Fig. 5.7.2., panel B). If the observed data is compared on a common axis (Fig. 5.7.3.) it is obvious that inhibition of PI 3-kinase activity does block M-CSF stimulated survival of BAC1.2F5 macrophages. However it is also evident that M-CSF alone does not potentiate survival of BAC1.2F5 macrophages and there is a degree of synergism between constituents of serum and M-CSF for the potentiation of BAC1.2F5 survival. PI 3-kinase activity does appear to be required for protection against apoptosis in BAC1.2F5 cells but it does not appear to be an absolute requirement. Since LPA has been shown to be a factor involved in macrophage survival it is possible that this is the serum constituent that is responsible for macrophage survival in addition to M-CSF. Investigation of LPA has revealed that it can act alone or in concert with M-

CSF to partially reduce apoptosis in cells cultured in low serum but does not block apoptosis completely which suggests that there is another factor in serum which is required for protection against apoptosis in BAC1.2F5 macrophages. This is borne out by the observation that M-CSF, recombinant LPA and delipidated serum together block apoptosis similar to levels seen in M-CSF and 10% serum treated cells. The data also indicates that M-CSF, LPA and the third serum factor or factors all have a positive effect on cell survival but only mediate their full effects together. Therefore, BAC1.2F5 cells differ from peritoneal macrophages, which are known to be poor proliferators (Yusoff et al., 1994), with regard to there requirement of serum factors for survival (Koh et al., 1998). In addition it is possible that other growth factors such as GM-CSF or even M-CSF itself which may be present in serum at very low concentrations.

In summary, these data indicate PI 3-kinase activity is:

(a) required for increases in cell numbers induced by M-CSF.

(b) not required for M-CSF stimulated proliferation.

(c) required to prevent cells undergoing apoptosis.

Thus the balance between the lack of increase in cell numbers when cells are stimulated with M-CSF in the presence of PI 3-kinase inhibitor is due to a preferential PI 3-kinase requirement for prevention of apoptosis, rather than a requirement for PI 3-kinase activation during the proliferation signal.

In addition several co-factors for survival and proliferation are necessary to obtain maximal increases in cell numbers in response to M-CSF. Lack of these factors leads to apoptosis. Inhibition of PI 3-kinase activity can overcome the presence of these survival factors leading to increases in apoptosis. Thus these survival factors are

likely to feed into a similar PI 3-kinase-dependent pathway. A combination of survival factors with M-CSF potentiates BAC1.2F5 survival and all are required before M-CSF can stimulate a proliferative signal. The balance between survival and proliferation is therefore mediated by separate signalling pathways downstream of c-*fms* in BAC1.2F5 macrophages, with PI 3-kinase activity required for protection against apoptosis and therefore cell survival.

CHAPTER 6

Effect of M-CSF on BAC1.2F5 cell morphology and vesicle formation is inhibited by PI 3-kinase inhibitors wortmannin and LY294002.

6.0.0. Introduction

Addition of M-CSF to BAC1.2F5 cells, quiesced for 24 hours in the absence of growth factor, stimulates rapid morphological changes including formation of lamellar processes and membrane ruffling within minutes of re-addition of growth factor (Boocock et al., 1989).

PI 3-kinase has been implicated in mediating growth factor stimulated vesicular membrane traffic. It is thought to mediate the earliest steps leading from ligation of cell surface receptors to increased cell surface ruffling. It was first demonstrated that PI 3-kinase activity was important for vesicle trafficking by the essential role for Vps34p, the yeast homolog of PI-specific 3-kinase, in late Golgi-vacuole trafficking in S. *cerevisiae* (Herman and Emr, 1990; Roth and Sternweis, 1997; Stack and Emr, 1994). Since PI-specific 3-kinase is homologous to Vps34p and so only produces one lipid product it has been proposed that it and its product, PI_3P, are important in the correct control of trans-golgi network-lysosome trafficking, late endosome-lysosome trafficking and endosomal recycling (Rameh et al., 1995). Additionally, $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$ the lipid products of the p85/p110 lipid kinase activity also appear to be involved in late endosome to lysosome trafficking and endosomal recycling (Downward, 1995). PI_3P and $PI_{3,4,5}P_3$ may have roles in vesicle trafficking, $PI_{3,4,5}P_3$ may activate an ARNO/cytohesin-1/Grp-1 family member to regulate the assembly of coated vesicles (Toker and Cantley, 1997).

The most compelling evidence in support of a role for PI 3-kinase activity during vesicle trafficking comes from studies conducted with PDGFr mutants (Zell et al., 1996). When the PI 3-kinase binding site on the PDGFr is mutated in fibroblasts blockage of PDGF-dependent receptor lysosomal degradation occurs (Shpetner et al.,

1996). Studies carried out in fibroblasts have shown that in the presence of wortmannin, trafficking of the internalised PDGF receptor is arrested at a juxtamembrane site, prior to reaching the sorting endosome (Shpetner et al., 1996).

Although wortmannin inhibits PI 3-kinase activity, causing an almost complete decrease in cellular levels of $PI_{3,4}P_2$ and $PI_{3,4,5}P_3$, there is only a 70% decrease in the levels of PI_3P (Shpetner et al., 1996). Any effects of wortmannin or LY 294002 at doses which only inhibit PI 3-kinase may preclude involvement of PI-specific 3-kinase products and are therefore useful tools for studying endocytotic events. Wortmannin and LY294002 have been shown to inhibit fluid-phase pinocytosis and Fc receptor mediated phagocytosis in macrophages, but with little effect on receptor mediated endocytosis of LDL (Araki et al., 1996), and so PI 3-kinase activity was therefore implicated in macropinocytosis and not micropinocytosis. In addition Araki *et al* have concluded that PI 3-kinase is necessary for closure of macropinosomes and phagosomes into intracellular organelles (Araki et al., 1996). Wortmannin also partially inhibits transferrin receptor appearance at the cell surface in CHO cells by increasing the internalisation rate and decreasing receptor recycling rate and hypervesiculation at the recycling compartment (Martys et al., 1996). Thus it appears that during receptor mediated endocytosis wortmannin sensitive enzymes are present at three stages; internalisation; transit from early endosomes to the recycling and lysosomal compartments and transit from recycling compartments to the cell surface activities which appear to be independent of PI_3P formation (Martys et al., 1996).

It is apparent that a function for PI 3-kinase activity remains to be clearly defined during vesicular transport particularly downstream of receptor mediated endocytosis in macrophages, a cell line which is particularly suited to this cellular response. The

aims of this results chapter were two fold. Firstly to characterise the effects of PI 3-kinase inhibitors on changes on vesicle formation and secondly to quantify the effects of PI 3-kinase inhibitors on cell morphology stimulated by M-CSF in the BAC1.2F5 macrophage cell line.

6.1.1. M-CSF stimulated morphological changes in BAC1.2F5 macrophages.

The effect of M-CSF on cell morphology was characterised by time-lapse video and photo-microscopy and quantified by calculating the percentage of cells containing vesicles in the total cell population. Prior to stimulation BAC1.2F5 cells were quiesced in the absence of M-CSF for 24 hours (T_0) (Fig. 6.1.1.). In the absence of M-CSF cells appear rounded with no discernible cellular structure, little or no membrane ruffling/activity and few if any intracellular vesicles (T_0) (Fig. 6.1.1.).

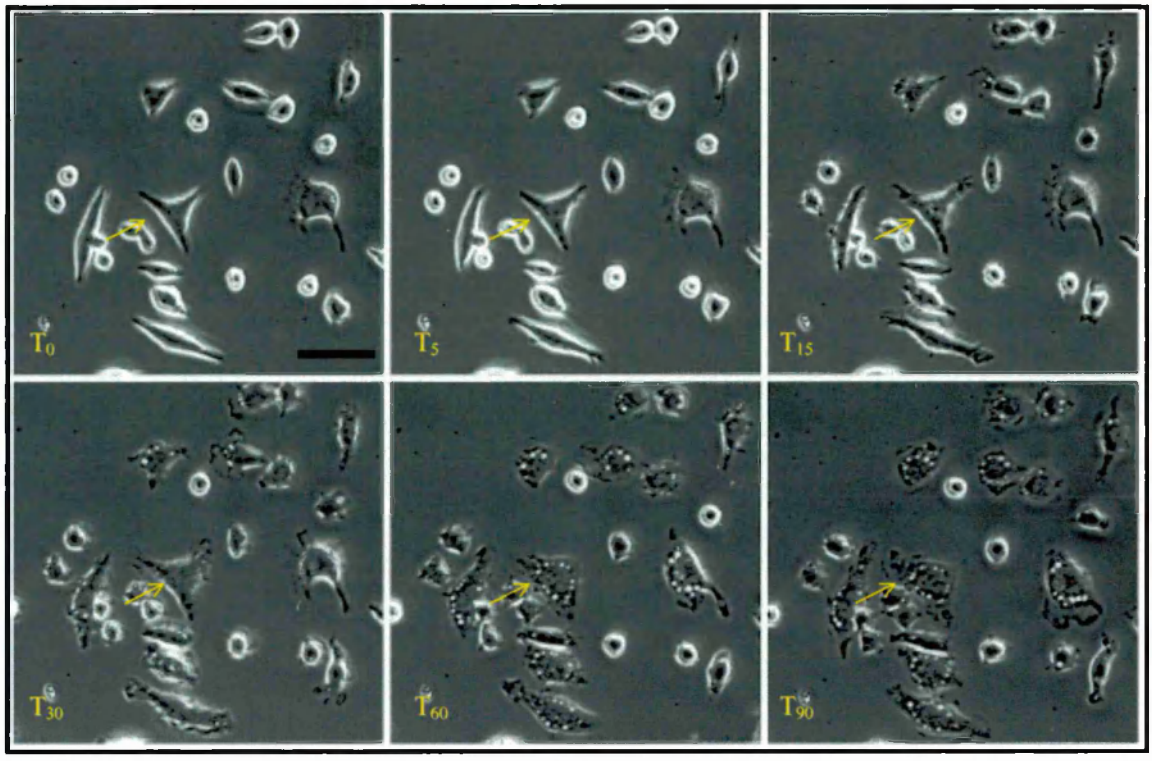


Fig. 6.1.1. Time-lapse photomicroscopy of the effect of M-CSF on BAC1.2F5 cells. Cells were quiesced for 24 hours in the absence of M-CSF (T_0), then stimulated with 100ng ml^{-1} M-CSF and recorded for 90 minutes by time-lapse photomicroscopy (T_5, T_{15}, T_{30}, T_{60} and T_{90}). This is a representative example of at least three independent experiments. Bar indicates 20μm.

Within 5 minutes (T_5) of M-CSF (100ng ml^{-1}) addition small vesicles begin to accumulate at the plasma membrane (see Chapter 7, Figs. 7.7.1 and 7.7.2), and they migrate towards the periphery of the nucleus within minutes of forming (Fig. 6.1.1., $T_{15} \rightarrow T_{30}$). The small vesicles tend to coalesce forming large macrovesicles which

remained at a peri-nuclear location for up to 90 minutes after addition of M-CSF (Fig. 6.1.1., T_{90}). Beginning 5 minutes after stimulation and continuing for up to 90 minutes the formation of vesicles is accompanied by cell spreading, pseudopod formation and upregulation of membrane activity including membrane ruffling which is associated with membrane internalisation (Fig. 6.1.1.). M-CSF stimulates rapid vesicle formation in BAC1.2F5 macrophages accompanied by increased membrane activity and cell spreading.

6.2.1. Effect of preincubation of LY294002 on M-CSF stimulated morphological changes in BAC1.2F5 macrophages

The role played by PI 3-kinase during M-CSF stimulated changes in cell morphology was investigated using the PI 3-kinase inhibitors wortmannin and LY294002. Wortmannin and LY294002 are specific inhibitors of PI 3-kinase activity at or near their IC_{50} values, (3nM and 1.4μM, respectively). Pre-incubation of BAC1.2F5 cells for 30 minutes with LY294002 at 1.4 μM inhibited M-CSF-mediated changes in cell morphology and vesicle formation (Fig. 6.2.1., top panel, T_{30}). In the presence of either 1.4μM or 10μM LY294002 there was far less vesicle formation than in untreated cells (Fig. 6.2.1., top and bottom panels, T_{30}). The reduction in vesicle formation was quantified and is presented in Fig. 6.2.3. Cells that contained vesicles at 30 minutes following M-CSF stimulation (T_{30}) possessed only a few, if any, vesicles by 60 minutes (T_{60}) whereas untreated cells contained more vesicles per cell (Fig. 6.2.1., top and bottom panels, T_{60}). In addition the rapid stimulation of membrane ruffling was partially inhibited by both concentrations of LY294002, resulting in a reduction of cell spreading and morphological changes which suggests

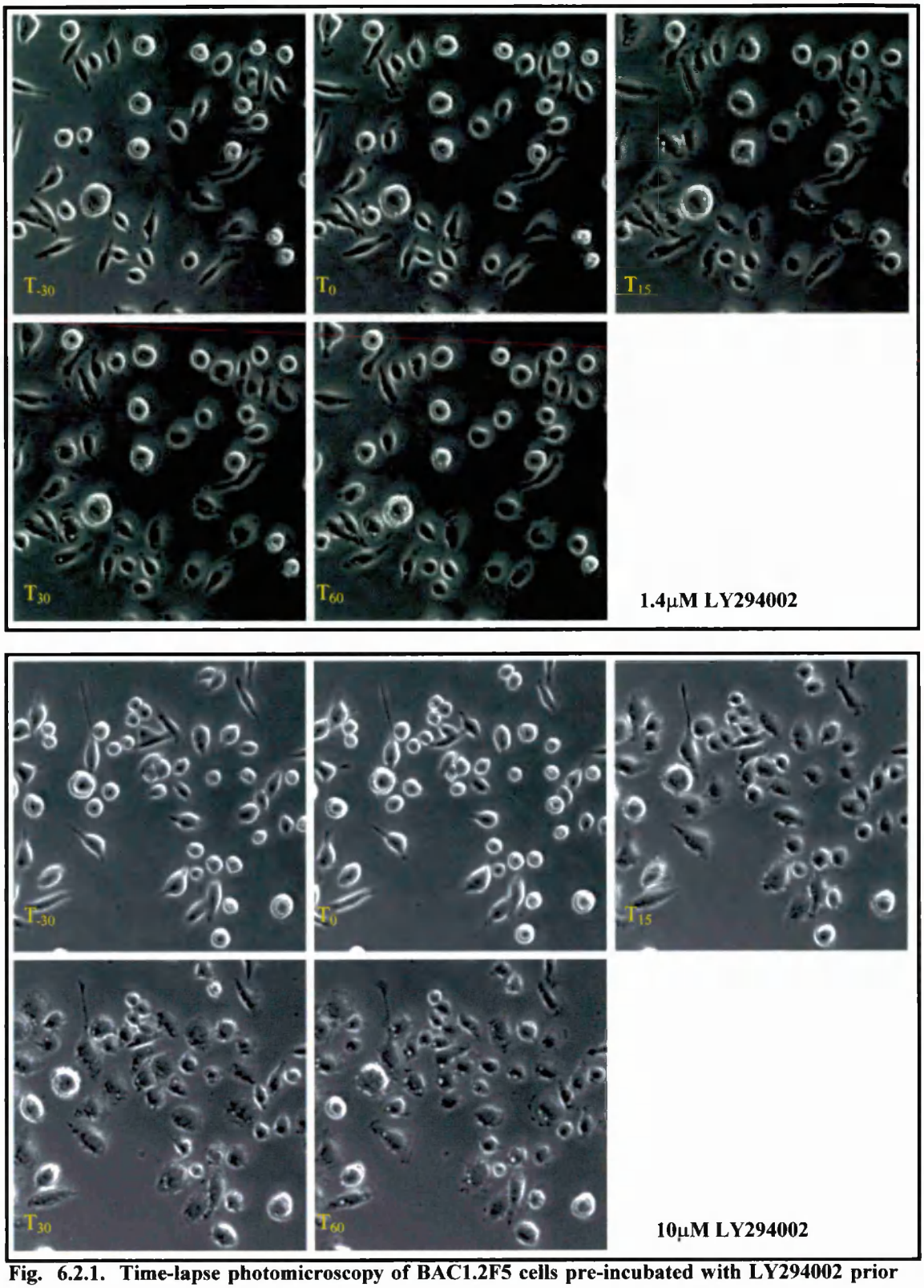


Fig. 6.2.1. Time-lapse photomicroscopy of BAC1.2F5 cells pre-incubated with LY294002 prior to stimulation with M-CSF. Cells quiesced for 24 hours were pre-incubated (T_{-30}) for 30 minutes with either 1.4μM (top panel) or 10μM LY294002 (bottom panel). Cells were then stimulated with 100ng ml^{-1} M-CSF (T_0) and recorded for a further 60 minutes (T_{30} and T_{60}). This is a representative example of at least three independent experiments.

that PI 3-kinase activity is also required for M-CSF stimulated actin rearrangement in BAC1.2F5 macrophages.

6.2.2. Effect of preincubation of wortmannin on M-CSF stimulated morphological changes in BAC1.2F5 macrophages

The effects of wortmannin were similar to LY294002 and pre-incubation of BAC1.2F5 cells for 30 minutes with wortmannin at either 3 or 30nM almost completely inhibited vesicle formation (T_{30}) and membrane ruffling (Fig. 6.2.2., top and bottom panel) after M-CSF stimulation. Although a proportion of the cell population did form vesicles, the vesicles did not migrate towards the nucleus and there was only a slight change in cell morphology but neither responses were comparable to M-CSF control (Fig. 6.1.1.).

6.2.3. Quantification of the effects of inhibitor pre-incubation on M-CSF stimulated vesicle formation

The effects of PI 3-kinase inhibitors wortmannin and LY294002 were quantified by counting the number of cells containing vesicles expressed as a percentage of the total cell population (Fig. 6.2.3.). Although the numbers of cells possessing vesicles at T_{30} was similar between untreated cells and cells treated with inhibitors, by 60 minutes (T_{60}) there was a substantial decrease in the numbers of cells containing vesicles (Fig. 6.2.3.). What is not apparent from the graph is that cells were scored positive if they contained at least one vesicle, and therefore does not represent the effect of LY294002 or wortmannin on vesicle formation within individual cells, i.e. the amount of vesiculation per cell.

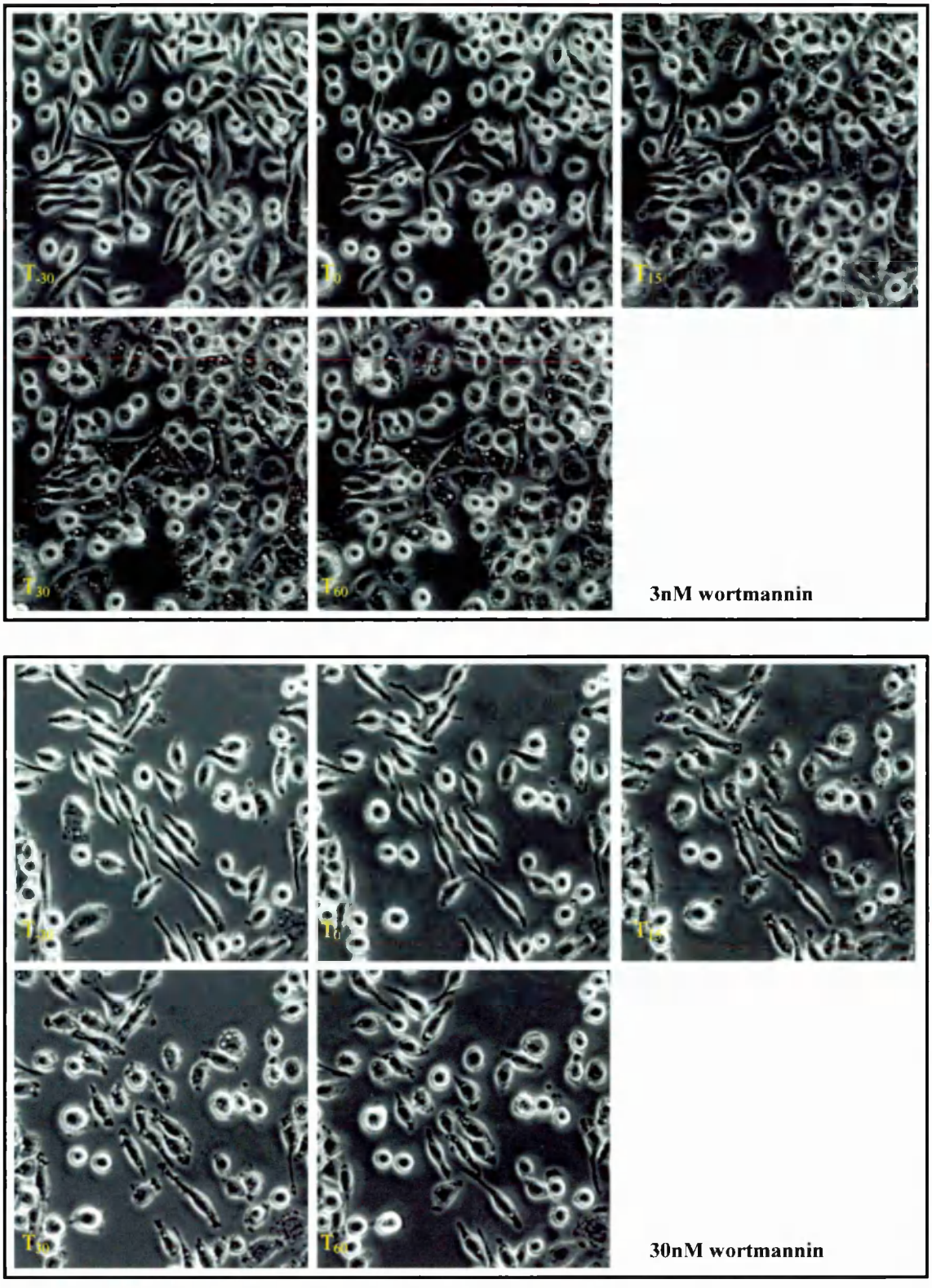


Fig. 6.2.2. Time-lapse photomicroscopy of BAC1.2F5 cells pre-incubated with wortmannin prior to stimulation with M-CSF. Cells quiesced for 24 hours were pre-incubated (T_{-30}) for 30 minutes with 3nM (top panel) or 30nM wortmannin (bottom panel). Cells were then stimulated with 100ng ml^{-1} M-CSF (T_0) and recorded for a further 60 minutes (T_{30} and T_{60}). This is a representative example of at least three independent experiments.

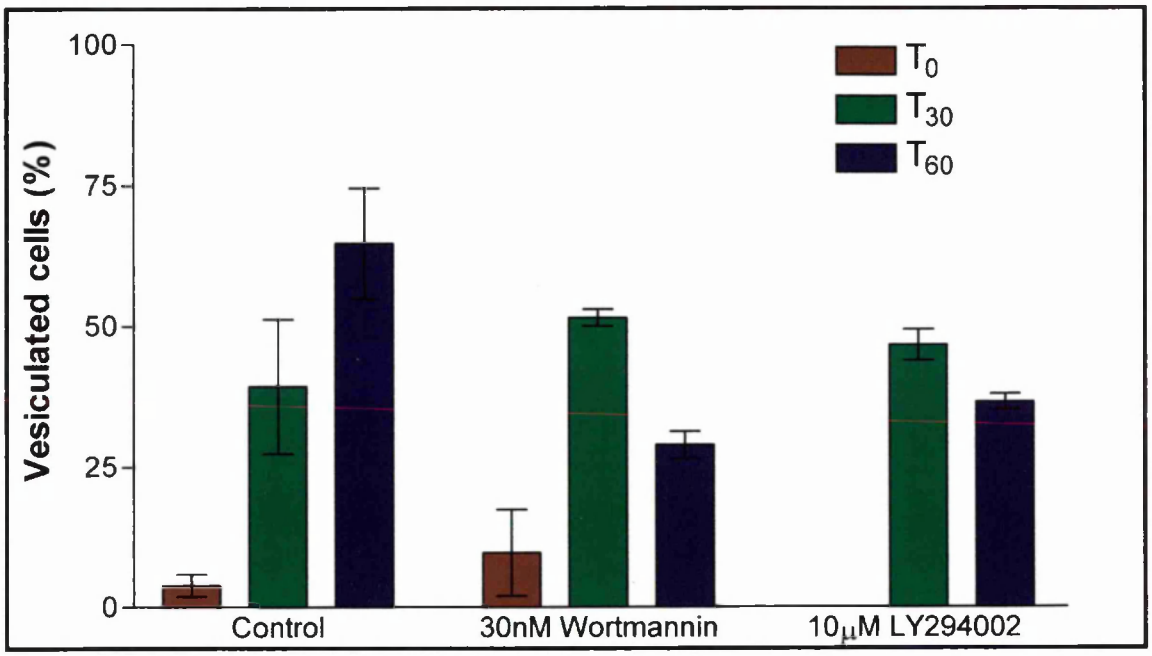


Fig. 6.2.3. Quantification of vesicle translocation in BAC1.2F5 cells stimulated with M-CSF prior to treatment with PI 3-kinase inhibitors. Photomicrographs were analysed and cells containing at least one vesicle were scored positive. Error bars are ±S.E.M (*n=3*).

6.3.1. Effect of LY294002 on morphological changes in BAC1.2F5 macrophages after M-CSF stimulation

To investigate whether M-CSF stimulated morphological responses and vesicle formation in BAC1.2F5 cells were reversible the PI 3-kinase inhibitors were added 30 minutes after stimulation with M-CSF. BAC1.2F5 cells were treated 30 minutes after M-CSF stimulation with LY294002 at 1.4μM or 10 μM (T_{30}). LY294002 at 1.4μM had no significant effect on M-CSF stimulated increases vesiculation or morphological changes in BAC1.2F5 cells (Fig. 6.3.1., top panel). However, treatment with 10μM LY294002 inhibited the changes in morphology and induced cell rounding, and M-CSF stimulated vesicles were no longer present by 60 minutes (Fig. 6.3.2., bottom panel, T_{60}).

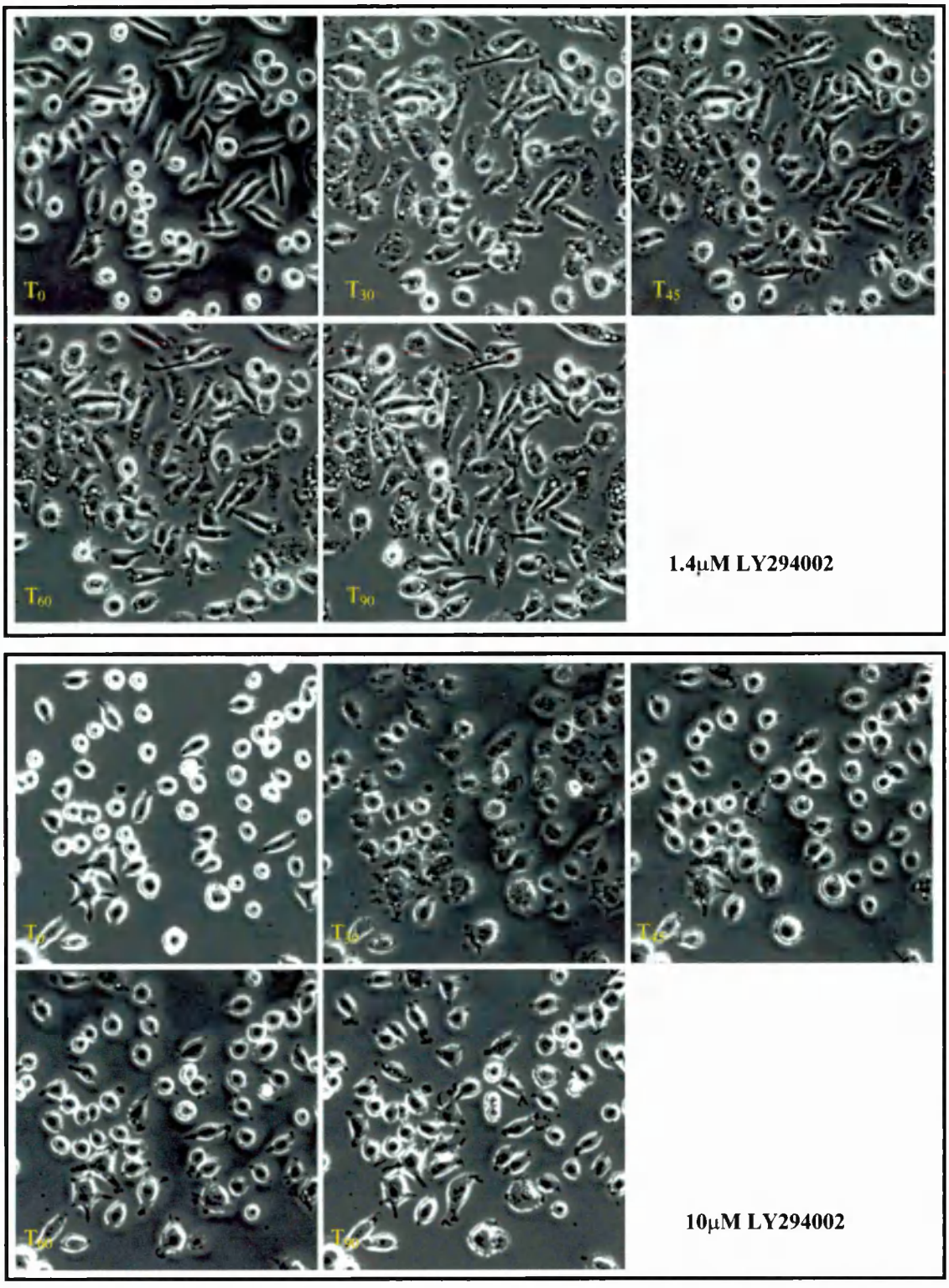


Fig. 6.3.1. Time-lapse photomicroscopy of BAC1.2F5 cells incubated with LY294002 after stimulation with M-CSF. Cells quiesced for 24 hours were stimulated for 30 minutes with 100ng ml^{-1} M-CSF (T_0). Cells were then treated (T_{30}) with either 1.4μM (top panel) or 10μM LY294002 (bottom panel) and recorded for a further 60 minutes (T_{60} and T_{90}). This is a representative example of at least three independent experiments.

6.3.2. Effect of wortmannin on morphological changes in BAC1.2F5 macrophages after M-CSF stimulation

At 3nM, wortmannin significantly reduced the effect of M-CSF on cell morphology (Fig. 6.3.2., top panel, T_{60}), and the proportion of the cell population containing vesicles was reduced by 60 minutes (Fig. 6.3.2., top panel, T_{60}). The effect of wortmannin was more pronounced by treatment with 30nM wortmannin (Fig. 6.3.2., bottom panel, T_{60}) which reversed the M-CSF stimulated vesicle formation observed at 30 minutes, prior to addition of wortmannin (Fig. 6.3.2., bottom panel, T_{30}). At 30nM, wortmannin also inhibited the M-CSF induced membrane ruffling and cell spreading (Fig. 6.3.2., bottom panel, T_{60}).

6.3.3. Quantification of the effects of inhibitors on vesicle formation after M-CSF stimulation

The effects of PI 3-kinase inhibitors wortmannin and LY294002 were quantified by counting the number of cells containing vesicles expressed as a percentage of the total cell population (Fig. 6.2.3.). Although the numbers of cells possessing vesicles at T_{30} was similar between untreated cells and cells treated with inhibitors, by 60 minutes (T_{60}) there was a substantial decrease in the numbers of cells containing vesicles (Fig. 6.2.3.). What is not apparent from the graph is that cells were scored positive if they contained at least one vesicle, and therefore does not represent the effect of LY294002 or wortmannin on vesicle formation within individual cells, i.e. the vesiculation per cell.

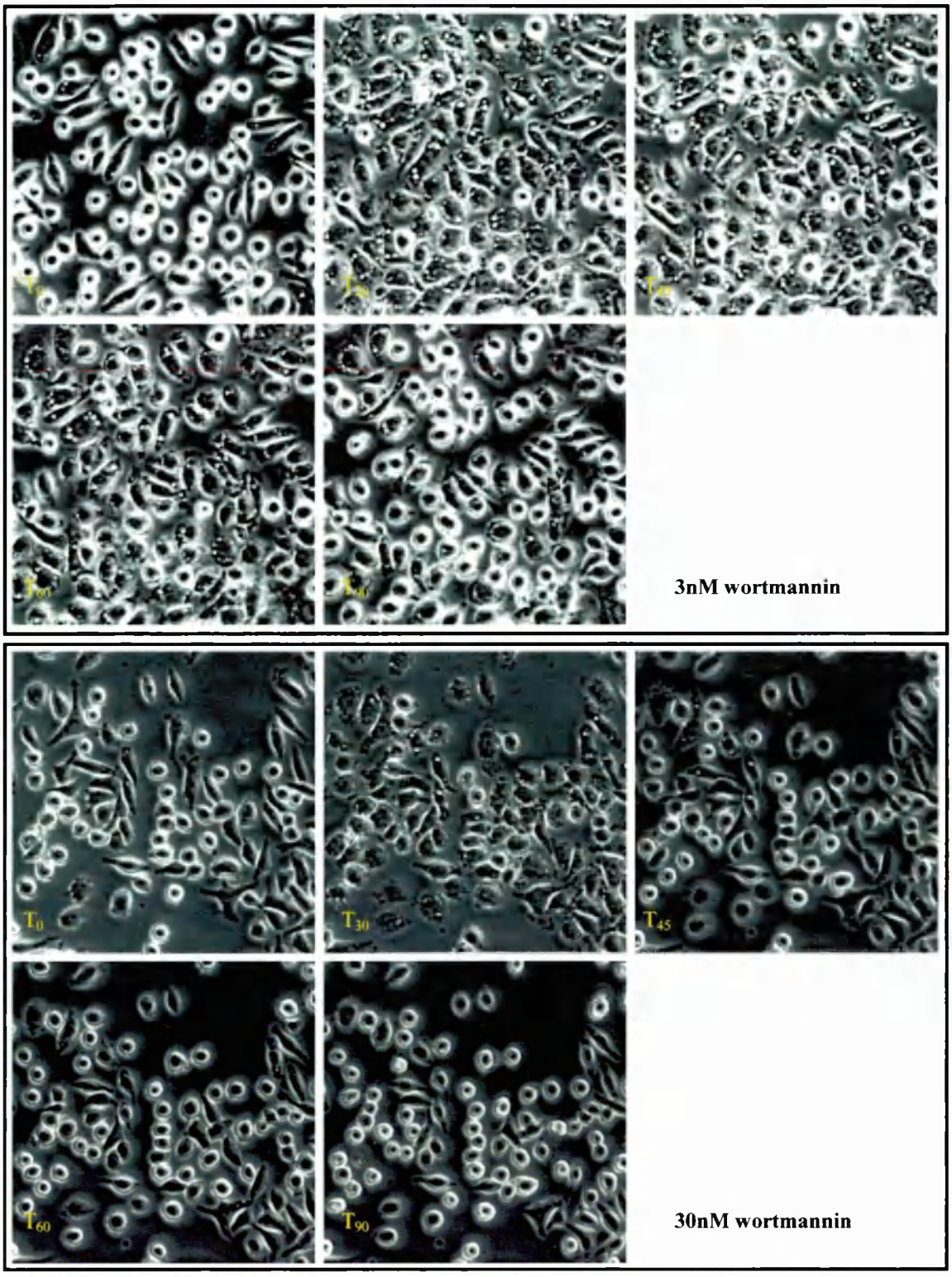


Fig. 6.3.2. Time-lapse photomicroscopy of BAC1.2F5 cells incubated with wortmannin after stimulation with M-CSF. Cells were quiesced for 24 hours then stimulated for 30 minutes with 100ng ml^{-1} M-CSF (T_0). Cells were then treated (T_{30}) with either 3nM or 30nM wortmannin. Cells were recorded for a further 60 minutes (T_{60} and T_{90}). This is a representative example of at least three independent experiments.

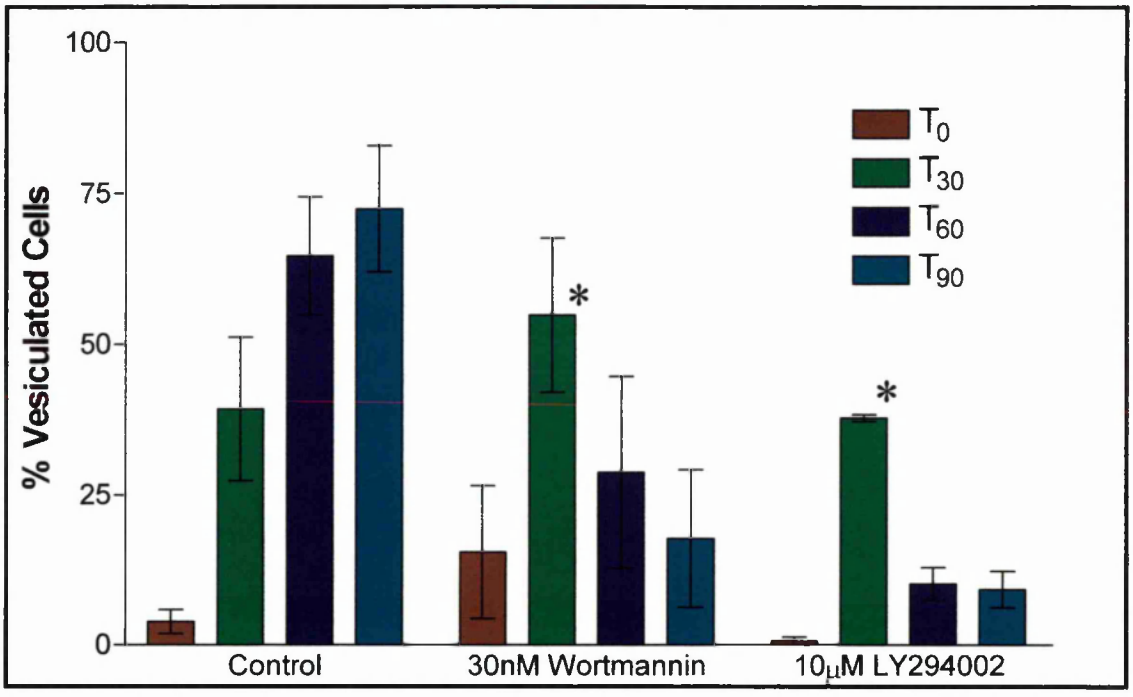


Fig. 6.3.3. Quantification of vesicle formation in BAC1.2F5 cells stimulated with M-CSF after treatment with PI 3-kinase inhibitors. Photomicrographs were analysed and cells containing at least one vesicle were scored positive. The time-point of inhibitor addition are indicated by the asterisks. Error bars are ±S.E.M (*n=3*).

6.4.0. Discussion

The response to M-CSF is characterised by various changes in the morphology of BAC1.2F5 cells. These morphological changes were orignally characterised by Boocock *et al* and have been determined to occur at concentrations of M-CSF which promote survival but not proliferation (Boocock et al., 1989). The addition of M-CSF to BAC1.2F5 cells stimulated the rapid formation of membrane ruffles and cell spreading which occurs after 15 minutes (Fig. 6.1.1.). After 30 minutes small punctate vesicles were present at the plasma membrane and in the newly forming lamellar processes which are characteristic of BAC1.2F5 cell spreading (Fig. 6.1.1.). After 60 minutes the cells were significantly more spread out than before M-CSF addtion and the small, punctate vesicles had coalesced to form larger vesicles, which have clustered around the nucleus of the cell, these vesicles persist for up to 90 minutes following M-CSF stimulation (Fig. 6.1.1.). The appearance of these large macrovesicles around the nucleus is similar to regulated macropinocytosis, which is different from clathrin-mediated endocytosis and is observed in many cells following stimulation, including macrophages (Swanson, 1989). Formation of macropinosomes is characterised by the formation of actin-rich ruffling at the periphery of the spreading cell and have been observed in M-CSF stimulated bone marrow derived macrophages (Racoosin and Swanson, 1989; Swanson, 1989). However, one of the characteristic features of macropinocytosis is that they are relatively short-lived in the cell, shrinking and disappearing after only 15 minutes (Swanson, 1989). Therefore, it is unlikely that internalisation of c-*fms* occurs via regulated macropinocytosis but more likely via clathrin-mediated endocytosis, which is a classical characteristic of receptor mediated endocytosis (Swanson, 1989).

The PI 3-kinase-specific inhibitors wortmannin and LY294002 were used to determine whether PI 3-kinase activity was involved in any of these M-CSF stimulated changes in BAC1.2F5 cells. Addition of the PI 3-kinase inhibitors to quiescent BAC1.2F5 cells before stimulation with M-CSF significantly reduced cell spreading, vesicle formation and membrane ruffling at concentrations which are believed to be specific for PI 3-kinase (Figs. 6.2.1. and 6.2.2.). The effects of LY294002 were more potent compared to wortmannin and may reflect the greater stability of LY294002, the half–life of wortmannin being only 30 minutes in culture (Vlahos et al., 1994). The formation of vesicles in M-CSF stimulated cells was scored on the presence or absence of cytosolic vesicle appearance (Fig. 6.2.3.). Although there was an initial increase in the number of cells containing vesicles in the presence of either wortmannin or LY294002, there were at least 50% less vesicle containing cells at 60 minutes post-stimulation than in M-CSF controls (Fig. 6.2.3.). Addition of the PI 3-kinase inhibitors after initial vesicle formation (30 minutes) inhibited further vesicle formation and cell spreading but no observed effect on membrane ruffling was observed (Figs. 6.3.1. and 6.3.2.). Inhibition of further vesicle formation and cell spreading appeared to be dose dependent and very rapid, occuring within 15 minutes of inhibitor addition (Figs. 6.3.1. and 6.3.2.). Quantification of vesicle formation in BAC1.2F5 cells treated with PI 3-kinase inhibitors following M-CSF stimulation confirmed that cells treated with inhibitors contained substantially less vesicles from 75% of cells in controls to less than 20% in the presence of inhibitors (Fig. 6.2.3.).

Together these data suggest that PI 3-kinase activity may not be required for the initial formation of endocytic vesicles, but may be required for their integrity and sub-cellular trafficking to downstream endosomal and/or lysosomal compartments in BAC1.2F5 macrophages. In addition wortmannin and LY294002 inhibited cell

spreading and membrane ruffling, two cytoskeletally controlled events which suggests that formation of PI 3-kinase products such as $PI_{3,4,5}P_3$ is important for reorganisation of the actin cytoskeleton possibly by regulation of the rho family GTPase, rac.

CHAPTER 7

Subcellular localisation of c-*fms* and PI 3-kinase in unstimulated and stimulated BAC1.2F5 macrophages.

7.0.0. Introduction

In unstimulated cells, c-*fms*, present at the cell surface, is turned-over with a half-life of 2-3 hours, followed by a period of several hours during which receptors re-accumulate at the cell surface following *de novo* synthesis (Sherr, 1988). When M-CSF binds to c-*fms*, a non-covalent receptor dimerisation event proceeds in conjunction with an initial wave of tyrosine phosphorylation on the receptor (Li and Stanley, 1991; Tapley et al., 1990). Receptor tyrosine dephosphorylation occurs in tandem with tyrosine phosphorylation of specific cytosolic proteins prior to extracellular, inter-chain disulphide linkage of receptor monomer subunits (Li and Stanley, 1991; Stanley et al., 1994). Inactivation of c-*fms* intrinsic kinase activity follows receptor dephosphorylation prior to modification via poly-ubiquitination and finally internalisation of the ligand-receptor complex (Stanley et al., 1994). Ubiquitination is a prerequisite for degradation of the c-*fms* cytoplasmic domain (Stanley et al., 1994). Internalisation of the ligand-receptor complex proceeds via clathrin coated vesicles, which are eventually transferred to secondary lysosomes for ligand-receptor degradation (Li and Stanley, 1991; Sherr and Stanley, 1990). Studies of a c-*fms* chimera, where the cytoplasmic tail of c-*fms* was fused to Glycophorin A suggest that there are structural features in the extracellular domain that determine targeting of internalised receptor to the lysosome for degradation (Lee and Nienhuis, 1992) Internalisation and degradation of c-*fms* down-regulates the M-CSF signal (Ohtsuka et al., 1990; Rettenmier et al., 1988). As stated in chapter 5, incubation of BAC1.2F5 cells at 4°C slows the rate of receptor degradation and can therefore be applied to the investigation of internalised c-*fms*. In addition, M-CSF stimulation of BAC1.2F5 cells also stimulated cell spreading, formation of filopodia and lamellapodia and reorganisation of the actin cytoskeleton (Allen et al., 1997; Boocock

et al., 1989).

The role of PI 3-kinase during internalisation of c-*fms* and its subsequent trafficking for degradation has not been studied; however inhibitors of PI 3-kinase block endocytosis in macrophages by blocking completion of vesicle formation, although initiation of vesicle formation still occurs (Araki et al., 1996). Wortmannin and LY294002 also inhibit fluid-phase pinocytosis and Fc receptor mediated phagocytosis, therefore PI 3-kinase activity is implicated in macropinocytosis (Araki et al., 1996). A role for PI 3-kinase activity during c-*fms* internalisation, or trafficking of c-*fms* containing vesicles has yet to be determined in macrophages.

$PI_{4,5}P_2$ and can bind to various proteins that regulate or bind to the actin cytoskeleton including gelsolin, cap-2, α-actinin and profilin and may serve as an attachment site for certain PH-domain containing proteins involved in regulation of the actin cytoskeleton (Bae et al., 1998; Rhee and Bae, 1997; Tapon and Hall, 1997). $PI_{4,5}P_2$ is a substrate for both PI 3-kinase and PLCγ, which has recently been demonstrated to be activated *in vitro* by $PI_{3,4,5}P_3$ (Bae et al., 1998). Therefore stimulation of PI 3-kinase results in activation of pathways which appear to have the net effect of depleting cellular $PI_{4,5}P_2$ levels, in turn affecting the organisation of the actin cytoskeleton.

The rho family proteins rho and rac are responsible for cell migration in response to M-CSF in the BAC1.2F5 macrophage cell line (Allen et al., 1998). It is known that PI 3-kinase is an effector for rho, since GTP-bound rho stimulates the production of PI 3,4,5-P_3 in platelet lysates (see below) (Stephens et al., 1993; Zhang et al., 1993). The other rho family member cdc42 is required for chemotactic response to M-CSF but not for locomotion (Allen et al., 1998). Allen *et al* have clearly defined the roles

of cdc42, rac and rho during M-CSF stimulated reorganisation of the cytoskeleton and cell adhesion in the BAC1.2F5 cell line (Allen et al., 1997). This suggests that PI 3-kinase products may be involved in stimulating rho activity and re-organisation of the actin cytoskeleton and cell locomotion. An example of this might be profilin which binds to $PI_{3,4,5}P_3$ and $PI_{4,5}P_2$ but more tightly to $PI_{3,4}P_2$ (Toker and Cantley, 1997). $PI_{3,4}P_2$ inhibits the ability of profilin to suppress actin polymerisation and the actin severing activity of gelsolin whilst stimulating actin uncapping in permeabilised platelets (Toker and Cantley, 1997). Although $PI_{4,5}P_2$ is the predominant lipid species found in cells, and is usually the relevant species that mediates actin assembly during activation of various growth factor receptors, considerable reorganisation of the actin cytoskeleton occurs, concomitant with the activation of PI 3-kinase, thus PI 3-kinase products may mediate the initial steps leading to increased membrane ruffling following activation of cell surface receptors such as those for PDGF and M-CSF (Araki et al., 1996).

Actin dynamics in lamellepodia are driven by continuous cycles of actin polymerisation, retrograde flow and actin depolymerisation (Welch et al., 1997). The leading edge of a motile cell or cells responding to a chemotactic gradient are composed of thin protrusions of membrane which continuously extend and retract, mediating the initial stage of cell movement whilst determining the direction of eventual movement (Welch et al., 1997). Growth factors such as PDGF stimulate actin reorganisation in fibroblasts and M-CSF stimulates actin reorganisation in BAC1.2F5 cells which leads to formation of filopodia, lamellepodia and membrane ruffles as well as fine actin cables within the cell. These changes in cell morphology are mediated by Rho family proteins that are regulated by a combination of GAPs,

GEFs and GDIs (Allen et al., 1997). Allen *et al* have demonstrated that formation of filopodia in response to M-CSF is mediated by cdc42 and formation of lamellepodia, membrane ruffling and focal adhesion complexes are mediated by rac which is also regulated indirectly by cdc42 and which also acts on rho leading to formation of actin cables (Allen et al., 1997). Interestingly, the formation of filopodia negatively regulates further lamellepodia, focal complex and actin cable formation as well as membrane ruffling. The role of PI 3-kinase in membrane ruffling has been demonstrated by the expression of a constitutively active p110α PI 3-kinase activity, which induced the formation of lamellepodia and focal complex formation and stress fibres in NIH 3T3 fibroblasts (Reif et al., 1996). However, PDGF-induced actin reorganisation is also blocked by mutation of a residue that is not involved in the direct binding of PI 3-kinase (Ruusala et al., 1998), which implies that multiple signals are necessary.

The main aims of this chapter were to characterise the subcellular localisation of c-*fms* and PI 3-kinase and the early time-course of c-*fms* internalisation in BAC1.2F5 macrophages by immunofluorescence. In addition the effects of M-CSF stimulation on the actin cytoskeleton were studied and the effects of the PI 3-kinase inhibitor LY294002 on c-*fms* internalisation and trafficking and the actin cytoskeleton was also characterised in BAC1.2F5 macrophages.

7.1.0. Internalisation of c-*fms* in BAC1.2F5 cells.

It has been reported in the literature that M-CSF is rapidly internalised into vesicles, through association with c-*fms*, upon stimulation of BAC1.2F5 cells (Boocock et al., 1989). However the subcellular localisation of c-*fms* itself during internalisation has not been examined to date and so the subcellular localisation of c-*fms* in BAC1.2F5 cells was examined by fluorescence microscopy. In unstimulated, quiescent BAC1.2F5 macrophages, c-*fms* staining is diffuse throughout the cytosol, with limited plasma membrane localisation, indicated by the arrows in Fig. 7.1.1. It is apparent that there are also differences in the amount of c-*fms* detected between quiescent cells although it has been reported that M-CSF starvation of BAC1.2F5 cells upregulates expression of c-*fms* (Boocock et al., 1989) (Fig. 7.1.1.). Stimulation of BAC1.2F5 cells with 50ng ml^{-1} M-CSF resulted in increased plasma membrane localisation of c-*fms* after 2 minutes (Fig. 7.1.2., arrow 1). In addition the accumulation of small, punctate areas of c-*fms* localisation were also observed in some cells, which tended to be localised on or near the plasma membrane and at the ends of lamellapodia, once formed (Fig. 7.1.2., arrow 2). After 20 minutes in the presence of M-CSF there was very little, if any, membrane staining (Fig. 7.1.3.). C-*fms* was now localised into ring-like structures in the cytosol of BAC1.2F5 cells (Fig. 7.1.3., arrow 1 and 2). These structures correspond to phase light vesicles which characteristically appear upon M-CSF stimulation of BAC1.2F5 cells. These vesicles are present throughout the cytosol and are found in the lamellepodia, and clustered around the nucleus (Fig. 7.1.3.). Most c-*fms* staining is absent 30 minutes after M-CSF stimulation and what staining is left is in a peri-nuclear location (Fig. 6.1.4., arrow). Therefore c-*fms* is internalised rapidly into vesicles which migrate towards the nucleus where it is degraded with 30 minutes.

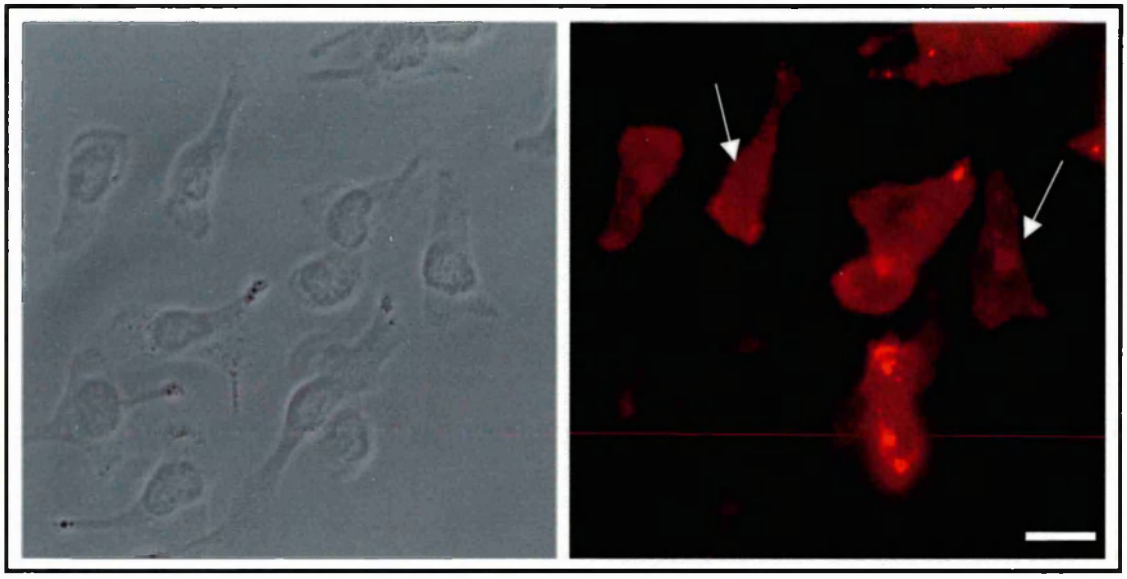


Fig. 7.1.1. Comparative phase contrast and fluorescent micrographs of BAC1.2F5 cells stained for c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours then probed with anti-c-*fms* mAb (Santa Cruz). Anti-c-*fms* antibody was detected by an Alexa Dye^{594}-conjugated secondary pAb (Molecular Probes). The left panel is a phase contrast image, the panel on the right is the same field of view stained for c-*fms*. Bar indicates 10μm.

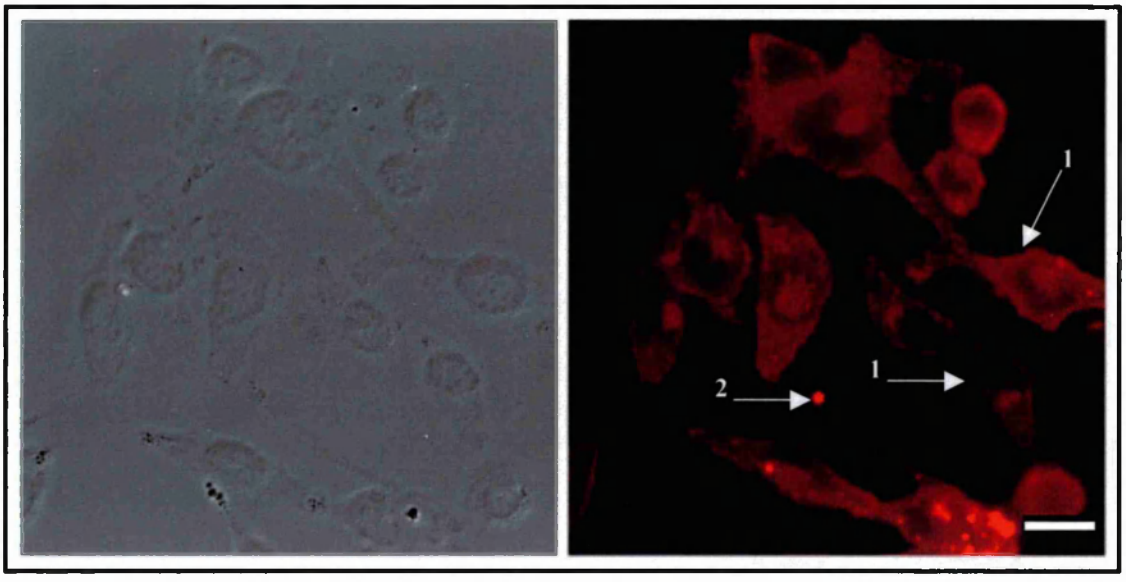


Fig. 7.1.2. Phase contrast and fluorescent micrographs of BAC1.2F5 cells stained for c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours then stimulated with 50ng ml^{-1} M-CSF for 2 minutes at 37°C before being fixed, permeabilised and probed with anti-c-*fms* mAb (Santa Cruz). Anti-c-*fms* antibody was detected by an Alexa Dye^{594}-conjugated secondary pAb (Molecular Probes). The left panel is a phase contrast image, the panel on the right is the same field of view stained for c-*fms*. Bar indicates 10μm.

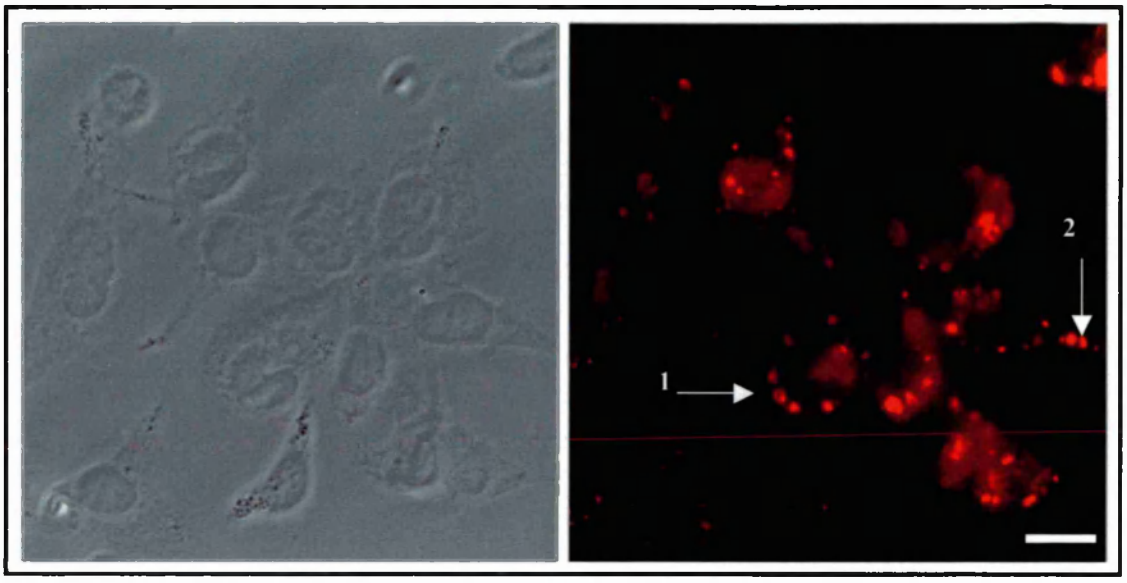


Fig. 7.1.3. Phase contrast and fluorescent micrographs of BAC1.2F5 cells stained for c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours then stimulated with 50ng ml^{-1} M-CSF for 20 minutes at 37°C before being fixed, permeabilised and probed with anti-c-*fms* mAb (Santa Cruz). Anti-c-*fms* antibody was detected by an Alexa Dye^{594}-conjugated secondary pAb (Molecular Probes). The left panel is a phase contrast image, the panel on the right is the same field of view stained for c-*fms*. Bar indicates 10μm.

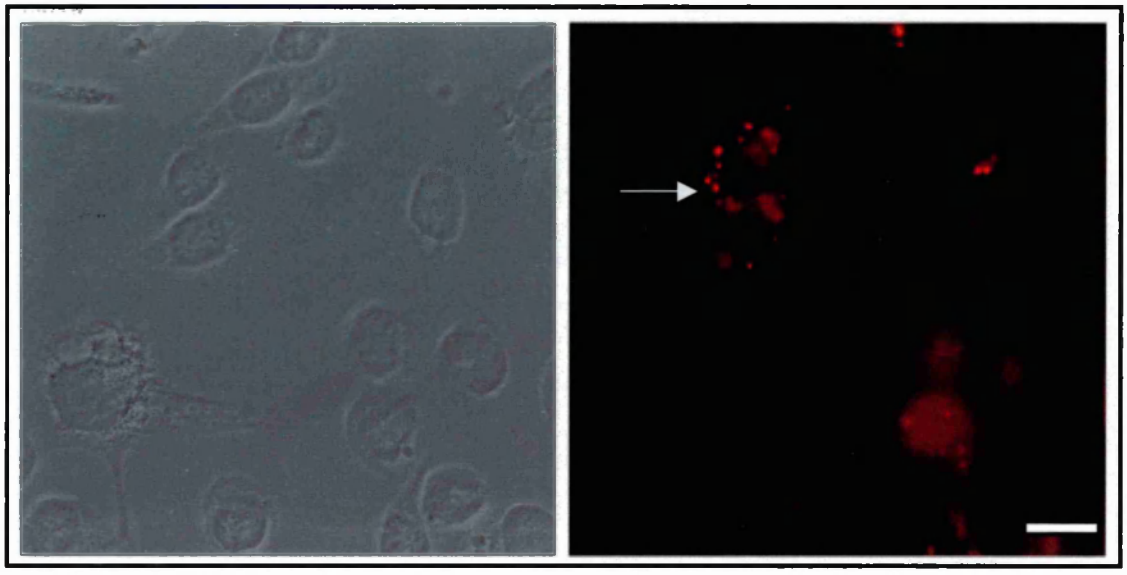


Fig. 7.1.4. Phase contrast and fluorescent micrographs of BAC1.2F5 cells stained for c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours then stimulated with 50ng ml^{-1} M-CSF for 30 minutes at 37°C before being fixed, permeabilised and probed with anti-c-*fms* mAb (Santa Cruz). Anti-c-*fms* antibody was detected by an Alexa Dye^{594}-conjugated secondary pAb (Molecular Probes). The left panel is a phase contrast image, the panel on the right is the same field of view stained for c-*fms*. Bar indicates 10μm.

7.2.0. Incubation of BAC1.2F5 cells at 4°C blocks receptor internalisation following stimulation with M-CSF

It has been reported that treatment of cells with M-CSF at 4°C slows down receptor internalisation as well as the very rapid increase in tyrosine phosphorylation of c-*fms* and various cytosolic proteins (Baccarini et al., 1991). In this case, incubation of BAC1.2F5 cells at 4°C during stimulation with M-CSF blocked receptor internalisation for at least 30 minutes, indicated by the heavy plasma membrane staining, and the absence of internalised receptor-bearing vesicles (Fig. 7.2.1.). This confirms that low temperature incubation of BAC1.2F5 cells slows down receptor internalisation and therefore receptor degradation.

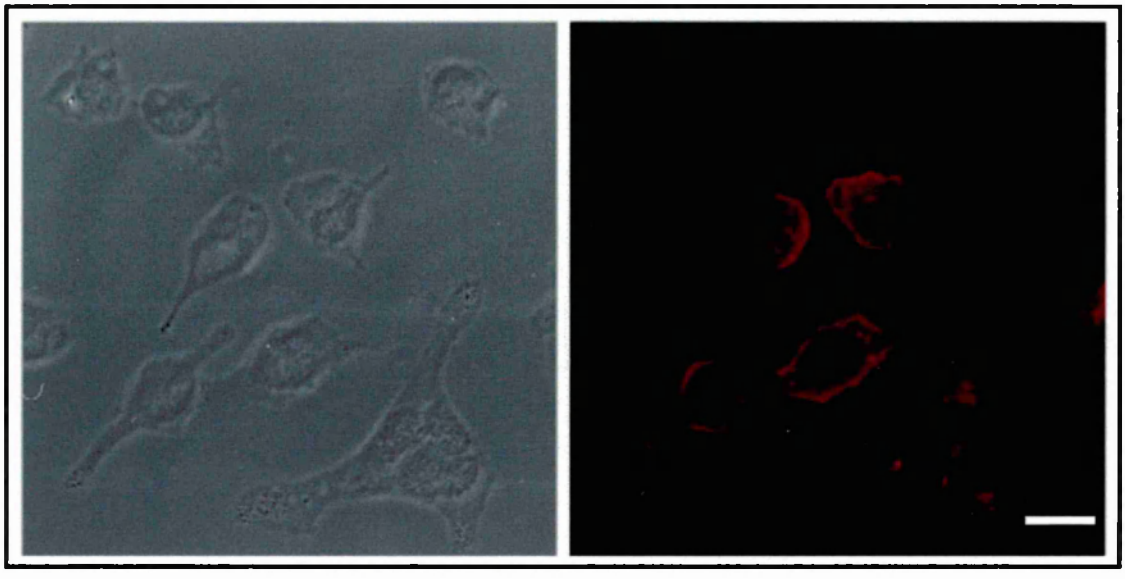


Fig. 7.2.1. Phase contrast and fluorescent micrographs of BAC1.2F5 cells stained for c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours then stimulated with 50ng ml^{-1} M-CSF for 30 minutes at 4°C before being fixed, permeabilised and probed with anti-c-*fms* mAb (Santa Cruz). Anti-c-*fms* antibody was detected by an Alexa Dye^{594}-conjugated secondary pAb (Molecular Probes). The left panel is a phase contrast image, the panel on the right is the same field of view stained for c-*fms*. Bar indicates 10μm.

7.3.0. Internalised c-*fms* is localised to phase light vesicles in BAC1.2F5 cells stimulated with M-CSF

Higher magnification of internalised c-*fms* at 10 minutes following M-CSF stimulation confirmed that the receptor staining was associated with phase light vesicles and this represented internalisation of the activated ligand-receptor complex (Fig. 7.3.1.).

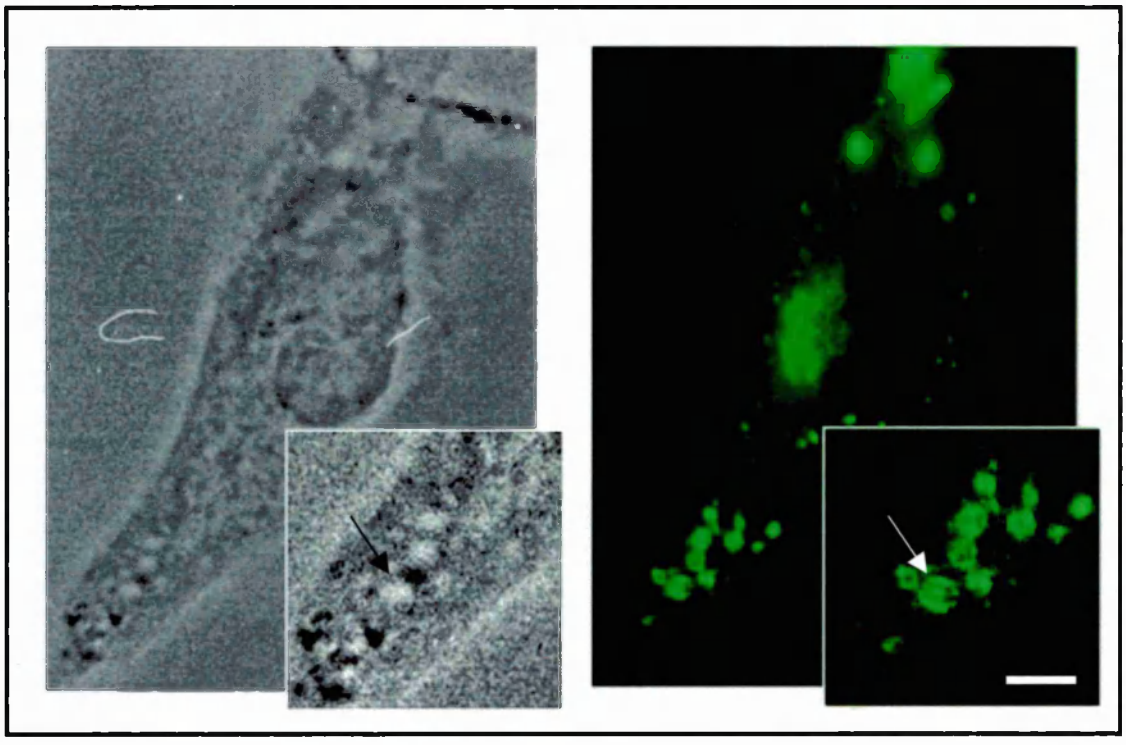


Fig. 7.3.1. Phase contrast and fluorescent micrographs of BAC1.2F5 cells stained for c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours then stimulated with 50ng ml^{-1} M-CSF for 10 minutes at 37°C before being fixed, permeabilised and probed with anti-c-*fms* mAb (Santa Cruz). Anti-c-*fms* antibody was detected by an Alexa Dye^{594}-conjugated secondary pAb (Molecular Probes). The left panel is a phase contrast image, the panel on the right is the same field of view stained for c-*fms*. Bar indicates 5μm.

In Fig. 7.3.1. the staining is found in the lamellar process which has formed following M-CSF stimulation. In addition the formation of slightly out of focus, small, punctate structures can be seen in the main body of the cell which are localised to the plasma membrane (Fig. 7.3.1.). The magnified inset illustrates that c-*fms* is localised to the membrane of the internalised vesicles, which vary in size (Fig. 7.3.1.). The arrows

indicate the position of a single internalised vesicle in both phase and fluorescent micrographs (Fig. 7.3.1. inset).

7.4.0. Tyrosine phosphorylation in BAC1.2F5 cells stimulated with M-CSF

In quiescent BAC1.2F5 cells there is a low basal level of tyrosine phosphorylation present in anti-phosphotyrosine western blots as illustrated in chapter 4.

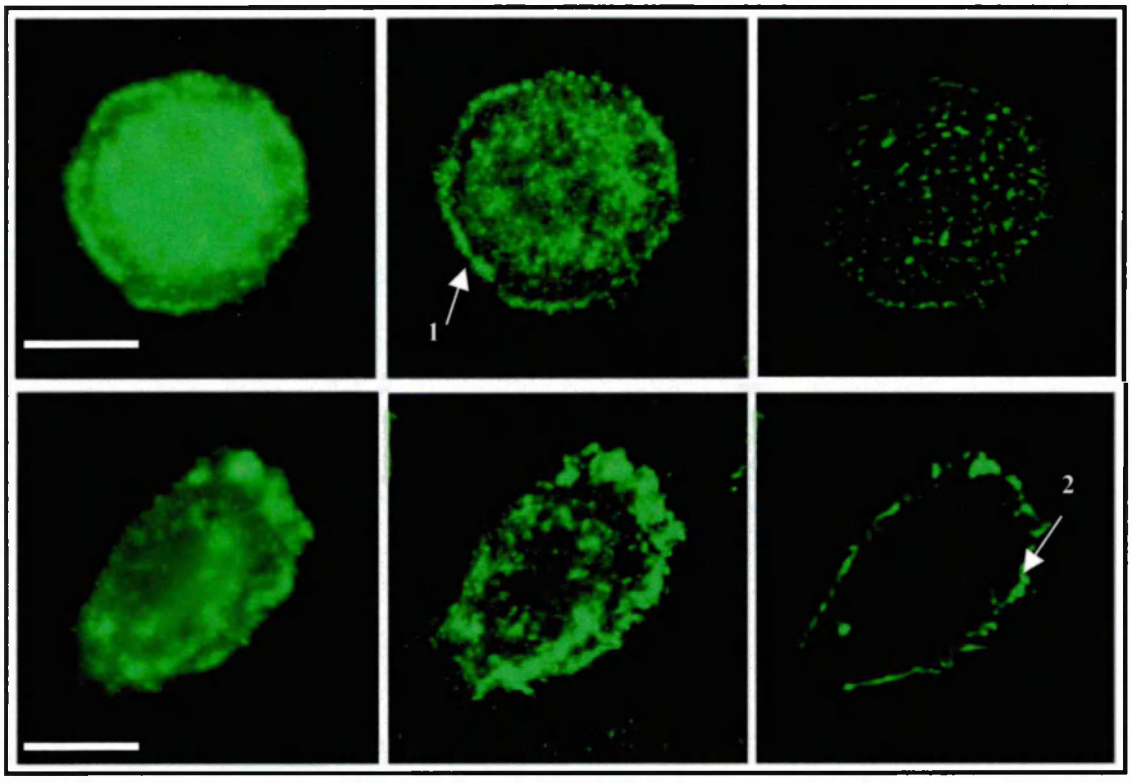


Fig. 7.4.1. Fluorescent micrographs of BAC1.2F5 cells stained for phosphotyrosine. BAC1.2F5 cells were quiesced for 24 hours then immediately stained (top panels), or stimulated with 50ng ml^{-1} M-CSF for 30 minutes at 4°C (bottom panels). Cells were then fixed, permeabilised and probed with an anti-pTyr mAb (Transduction Labs.). Anti-pTyr antibody was detected by an FITC-conjugated secondary pAb (Sigma). Images were digitally captured and analysed with Biovision and MacHazeBuster (Improvision and Vaytek) The panels on the left are composite images prior to deconvolution, the middle panels are the same composite images after deconvolution and the panels on the right are a single 1μm section from the deconvolved composite image. Bar indicates 10μm.

In the absence of M-CSF there is a low basal level of tyrosine phosphorylation throughout the BAC1.2F5 cell (Fig. 7.4.1., top panels). Tyrosine phosphorylation

appears punctate throughout the cytosol with some staining present at the plasma membrane (Fig. 7.4.1., top panel, arrow 1). Stimulation of quiescent BAC1.2F5 cells for 30 minutes at 4°C with M-CSF stimulated recruitment of tyrosine phosphorylated proteins to the plasma membrane with a concomitant reduction in the levels of cytosolic proteins phosphorylated on tyrosine (Fig. 7.4.1., bottom panel, arrow 2). This suggested that in BAC1.2F5 cells, M-CSF stimulation results in the recruitment of tyrosine phosphorylated proteins to the plasma membrane.

7.5.0 Localisation of p85α and p110α in M-CSF stimulated cells

Recruitment of PI 3-kinase activity has been shown to be important to c-*fms* signalling. Therefore the subcellular localisation of the PI 3-kinase subunits p85α and p110α were investigated. In BAC1.2F5 cells cultured in the absence of M-CSF, p85α staining is mainly cytosolic (Fig. 7.5.1., bottom right panel, arrow 1)., although some plasma membrane staining is seen in the deconvolved section (Fig. 7.5.1., bottom right panel, arrow 2). Stimulation of quiescent BAC1.2F5 cells for 30 minutes at 4°C with M-CSF stimulated recruitment of p85α to the plasma membrane (Fig. 7.5.1., top panels).

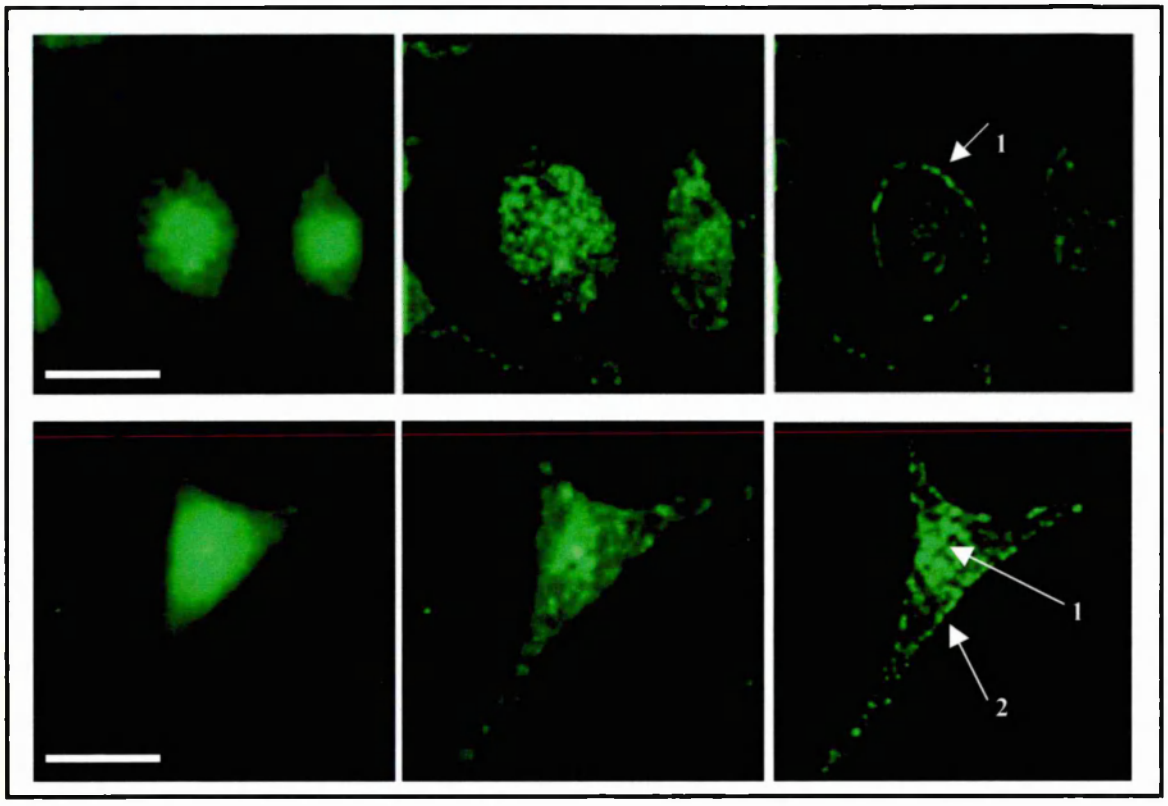


Fig. 7.5.1. Fluorescent micrographs of BAC1.2F5 cells stained for p85α. BAC1.2F5 cells were quiesced for 24 hours then immediately stained (bottom panels), or stimulated with 50ng ml^{-1} M-CSF for 30 minutes at 4°C (top panels). Cells were then fixed, permeabilised and probed with an anti-p85α mAb (Transduction Labs.). Anti-p85α antibody was detected by an FITC-conjugated secondary pAb (Sigma). Images were digitally captured and analysed with Biovision and MacHazeBuster (Improvision and Vaytek) The panels on the left are composite images prior to deconvolution, the middle panels are the same composite images after deconvolution and the panels on the right are a single 1μm section from the deconvolved composite image. Bar indicates 10μm.

BAC1.2F5 cells cultured in the presence of M-CSF showed predominantly cytosolic staining for the p110α subunit (Fig. 7.5.2., top panels, arrow 1). However, stimulation of quiescent BAC1.2F5 cells for 30 minutes at 4°C with M-CSF stimulates recruitment of p110α to the plasma membrane with a concomitant reduction in the levels of p110α found in the cytosol (Fig. 7.5.2., bottom panels, arrow 2). This suggests that in BAC1.2F5 cells, M-CSF stimulation results in the recruitment of both p85α and p110α to the plasma membrane which is required for full PI 3-kinase activation.

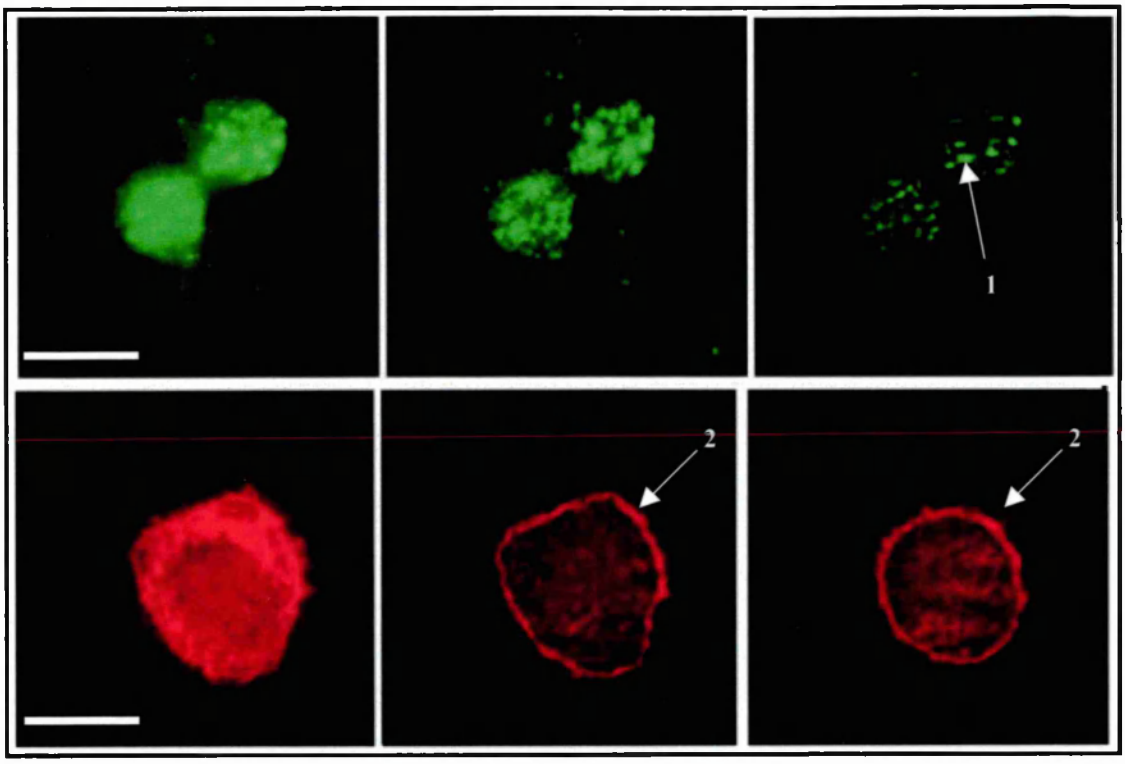


Fig. 7.5.2. Fluorescent micrographs of BAC1.2F5 cells stained for p110α. BAC1.2F5 cells were either cultured in normal medium containing M-CSF (Top panels) or in the absence of M-CSF for 24 hours before stimulation with 50ng ml^{-1} M-CSF for 30 minutes at 4°C (Bottom panels). Cells were then fixed, permeabilised and probed with an anti-p110α mAb (Transduction Labs.). Anti-p110α antibody was detected by either a TRITC- or FITC-conjugated secondary pAb (Sigma). Images were digitally captured and analysed with Biovision and MacHazeBuster (Improvision and Vaytek). The panels on the left are composite images prior to deconvolution, the middle top panel is the same composite image as top left after deconvolution and middle bottom and the panels on the right are single 1μm sections from the deconvolved composite images. Bar indicates 10μm.

7.6.0 The BAC1.2F5 actin cytoskeleton

Stimulation of quiescent BAC1.2F5 cells has been reported to stimulate the rapid reorganisation of the actin cytoskeleton, which is important for maintainence of cell morphology (Allen et al., 1997). The M-CSF stimulated changes in actin organisation was investigated in BAC1.2F5 macrophages over the early time-course concurrent with c-*fms* internalisation.

7.6.1. M-CSF stimulated reorganisation of the BAC1.2F5 actin cytoskeleton

In normal culture conditions BAC1.2F5 cells exhibit organised actin staining with marked staining in the cell leading edge (Fig. 7.6.1., panel N, arrow 1). Throughout the cytoplasm small, punctate actin staining was observed and digital deconvolution revealed that this was predominantly present at the ventral face of the cell (Fig. 7.6.1., panel N, arrow 2), in contact with the glass coverslip, presumably corresponding to podosomes (Dutartre et al., 1996; Gavazzi et al., 1989). BAC1.2F5 cells quiesced for 24 hours in the absence of M-CSF were noticeably rounder in appearance and had similar actin organisation to cells in normal culture with heavy plasma membrane staining (Fig. 7.6.1., panel US, arrow 1) and some cytosolic, punctate staining that was present on the cell membrane, in contact with the glass coverslip (Fig. 7.6.1., panel US, arrow 2).

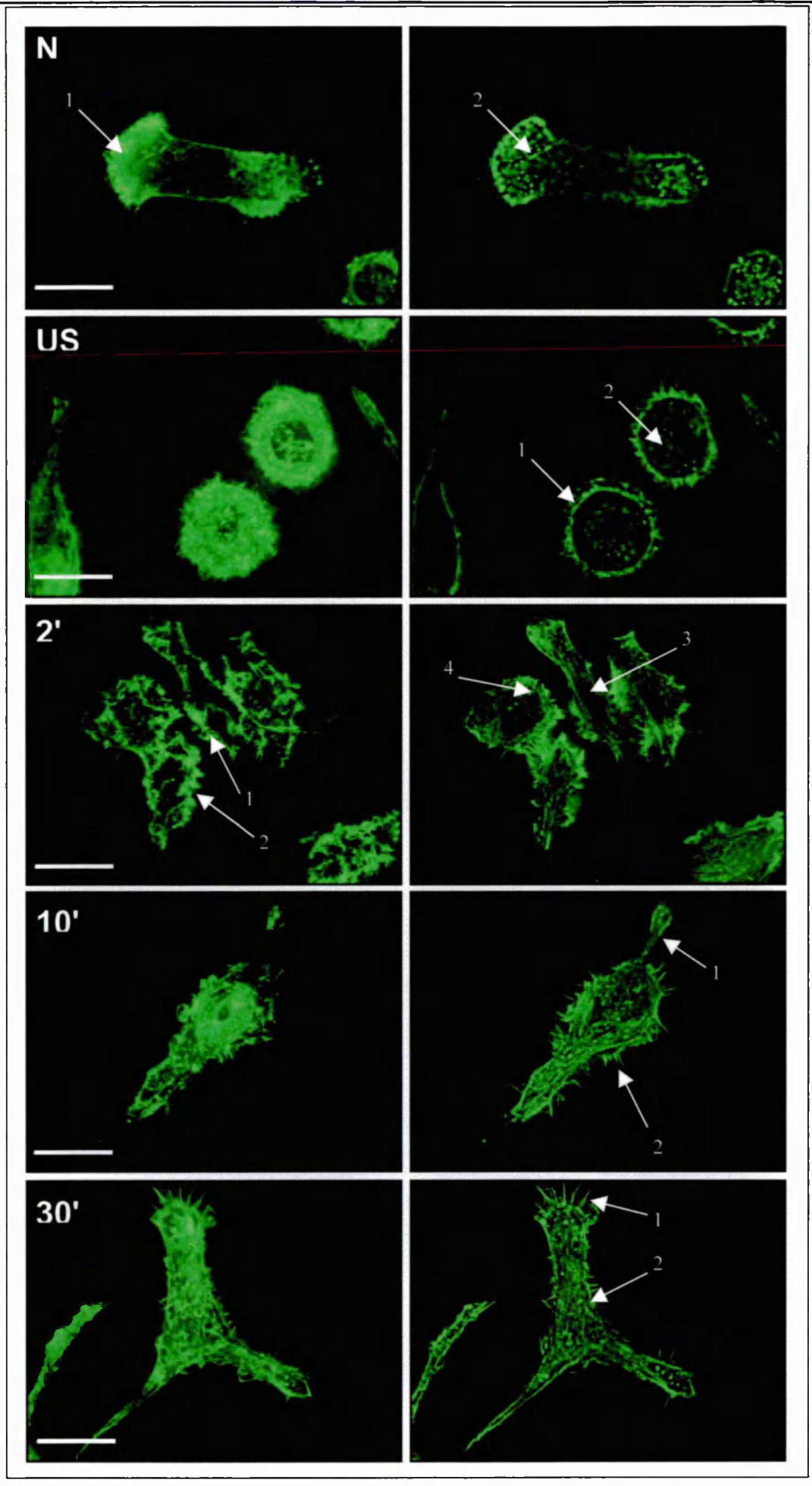
Stimulation of BAC1.2F5 cells with M-CSF resulted in dramatic changes in cell morphology which were characterised by reorganisation of the actin cytoskeleton. Cells stimulated for 2 minutes with 50ng ml^{-1} M-CSF had increased actin staining in plasma membrane folds, indicative of membrane ruffles (Fig. 7.6.1., panel 2', arrow 1). In addition small actin-rich filopodia were also present in some, but not all cells

(Fig. 7.6.1., panel 2', arrow 2). Within the cytoplasm of BAC1.2F5 cells major reorganisation of the actin cytoskeleton was characterised by the formation of fine actin cables that organised themselves longitudinally throughout polarised cells (Fig. 7.6.1., panel 2', arrow 3) and in concentric rings in rounded cells (Fig. 7.6.1., panel 2', arrow 4).

After 10 minutes, long, lamellapodia had formed in the majority of the cells with some cells containing more than one lamellapodia (Fig. 7.6.1., panel 10', arrow 1). In addition the majority of BAC1.2F5 cells contained abundant actin-rich filopodia and membrane ruffles. The cytoplasmic cytoskeleton remained well organised and the fine actin cabling was still apparent (Fig. 7.6.1., panel 10', arrow 2). These features of the actin cytoskeleton, present at 10 minutes following M-CSF stimulation were also observed at 30 minutes, with no observable differences (Fig. 7.6.1., panel 30', arrows 1+ 2). Incubation with M-CSF for 30 minutes at 4°C blocked the reorganisation of the actin cytoskeleton and there was no formation of actin-rich membrane ruffles, filopodia or actin cables (data not shown).

On the following page:

7.6.1. Fluorescent micrographs of BAC1.2F5 cells stained for polymerised actin. BAC1.2F5 cells were cultured without M-CSF for 24 hours before stimulation with 50ng ml^{-1} M-CSF. Cells were treated as follows; normal culture cells (N), unstimulated (US), stimulated for 2 minutes (2'), 10 minutes (10') or 30 minutes (30'). Cells were then fixed, permeabilised and stained with a phalloidin-FITC conjugate (Sigma). Images were digitally captured with an OpenLab digital image analysis system. Left hand panels are reconstruction of 25 composite, 0.5μm sections after software deconvolution. The right hand panels are the same field of view but are a single representative section. Bar indicates 10μm.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At higher magnification, the formation of filopodia and membrane ruffles are clearly visible (Fig. 7.6.2.). After 5 minutes in the presence of M-CSF actin-rich filopodia extend from the plasma membrane and radiate out from the cell (Fig. 7.6.2., panel A, arrows). In parallel , actin-rich membrane ruffles also appear and are present over the entire surface of the cell (Fig. 7.6.2., panel B, arrows).

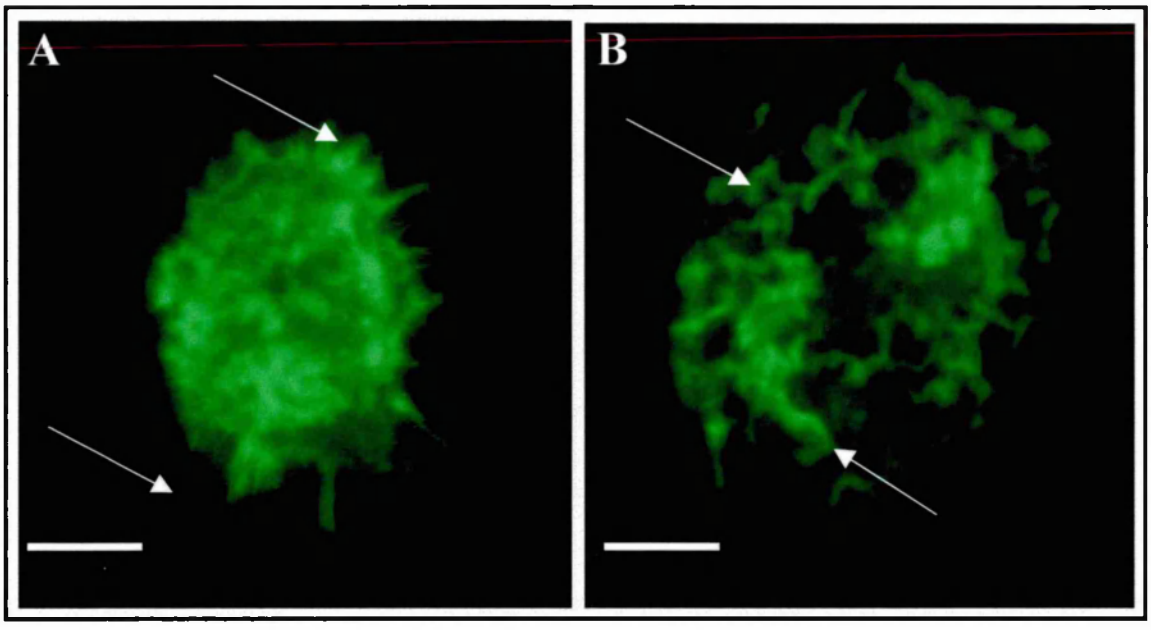


Fig. 7.6.2. Fluorescent micrographs of BAC1.2F5 cells stained for polymerised actin. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours before stimulation with 50ng ml^{-1} M-CSF. Cell were stimulated for 5 minutes at 37°C then fixed, permeabilised and stained with a phalloidin-FITC conjugate (Sigma). Images were digitally captured with Biovision image capture software (Improvision). Bar indicates 5μm

Closer examination of the formation of fine actin cables in M-CSF stimulated BAC1.2F5 cells was investigated using the OpenLab digital image capture system. Following a 10 minute stimulation with M-CSF the formation of fine actin cables within the cytoplasm were clearly visible and appeared to align longitudinally along the polarised axis of the cell , c-*fms* staining is shown for comparison (Fig. 7.6.3.). In the composite image of Fig. 7.6.3. there is a limited amount of c-*fms* containing vesicles which appear to align with the actin cables. This may represent the transport

of internalised, clathrin-coated c-*fms* containing vesicles from the cell periphery or lamellepodia tips, towards the endosomal and lysosomal compartments in BAC1.2F5 macrophages (Fig. 7.6.3., merged, arrows).

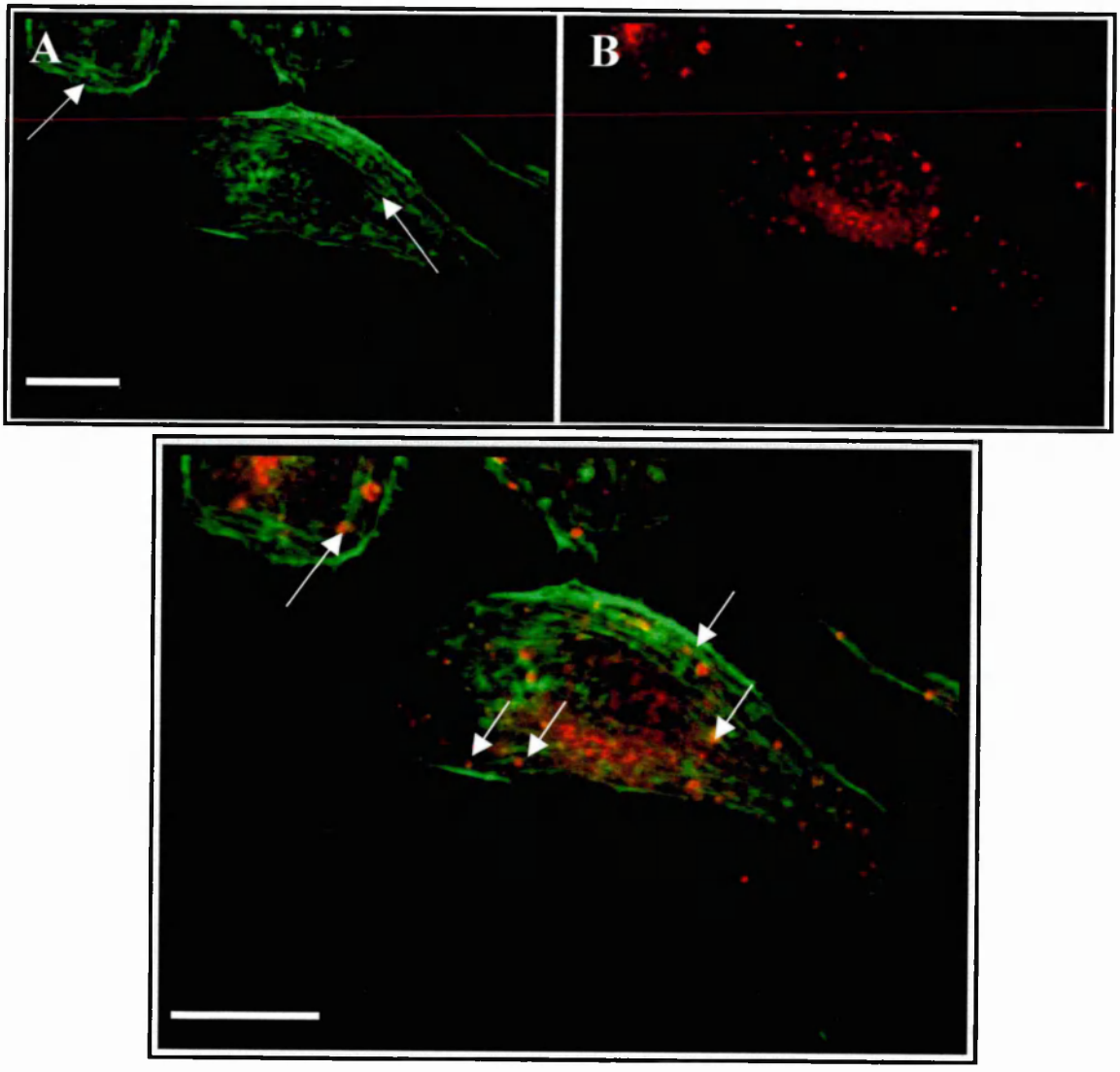


Fig. 7.6.3. Fluorescent micrographs of BAC1.2F5 cells stained for polymerised actin and c-*fms*. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours before stimulation for 10 minutes with 50ng ml^{-1} M-CSF. Cells were then fixed, permeabilised and stained with anti-c-*fms* antibody (Santa Cruz) and counter-stained with a phalloidin-FITC conjugate (Sigma). Images were digitally captured and analysed with OpenLab 1.7.8. Panel A represents F-actin staining and panel B represents the same field of view stained for c-*fms*. The bottom panel is a composite of panel A and B. Bar indicates 10μm.

7.6.4. Effect of LY294002 on the M-CSF stimulated reorganisation of the BAC1.2F5 actin cytoskeleton

In BAC1.2F5 cells cultured under normal conditions addition of LY294002 induced the formation of actin-rich retraction fibres in the majority of cells (Fig. 7.6.4., panel N, arrows 1 and 2). Throughout the cytoplasm small, punctate actin staining was observed and digital deconvolution revealed that this was predominantly present on the cell membrane, in contact with the glass coverslip but the majority of actin staining was at the plasma membrane (Fig. 7.6.4., panel N, arrow 2). In addition the organised actin cytoskeleton, observed in untreated cells was absent.

BAC1.2F5 cells quiesced for 24 hours in the absence of M-CSF were noticeably rounder in appearance and had similar actin organisation to untreated, unstimulated cells. In cells that had remained polarised addition of LY2940002 had induced formation of retraction fibres (Fig. 7.6.1., panel US, arrow 1). Actin staining was predominantly found at the plasma membrane (Fig. 7.6.4., panel US, arrow 2) as well some punctate cytosolic staining.

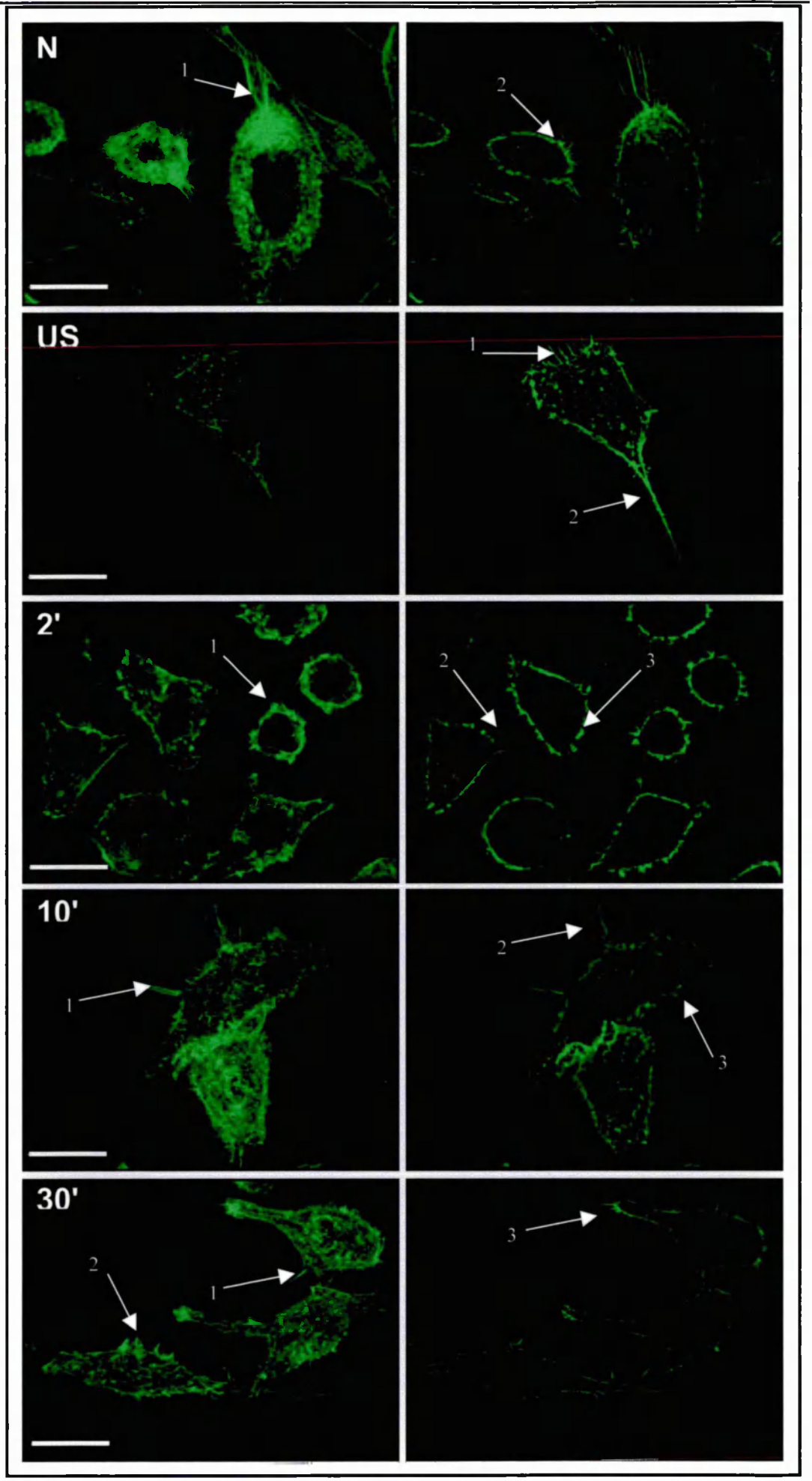
Stimulation of BAC1.2F5 cells with M-CSF still induced dramatic changes in cell morphology, characterised by the formation of actin-rich membrane ruffles (Fig. 7.6.4., panel 2', arrow 1). Actin-rich filopodia were present in some (Fig. 7.6.4., panel 2', arrow 2), but not all cells, however the majority of actin staining was present at the plasma membrane (Fig. 7.6.4., panel 2', arrow 3). The reorganisation of actin into fine cables was inhibited by LY294002 (Fig. 7.6.4., panel 2').

After 10 minutes retraction fibres were still present (Fig. 7.6.4., panel 10', arrow 1) as

were the M-CSF stimulated membrane ruffles and predominant plasma membrane staining (Fig. 7.6.4., panel 10', arrows 2 and 3). After 30 minutes, There was little change in the actin organisation in the presence of LY294002, with some membrane ruffles (Fig. 7.6.4., panel 30', arrows 1 and 2), predominant plasma membrane staining (Fig. 7.6.4., panel 30', arrow 3), but no actin cable formation.

On the following page:

7.6.4. Fluorescent micrographs of BAC1.2F5 cells stained for polymerised actin. BAC1.2F5 cells were cultured without M-CSF for 24 hours before stimulation with 50ng ml^{-1} M-CSF. Cells were pre-incubated with 10μM LY294002 for 10 minutes then treated as follows; normal culture cells (N), unstimulated (US), stimulated for 2 minutes (2'), 10 minutes (10') or 30 minutes (30'). Cells were then fixed, permeabilised and stained with a phalloidin-FITC conjugate (Sigma). Images were digitally captured with an OpenLab digital image analysis system. Left hand panels are reconstruction of 25 composite, 0.5μm sections after software deconvolution. The right hand panels are the same field of view but are a single representative section. Bars indicate 10μm.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At higher magnification, LY294002 induced retraction fibres up to 15μm in length were clearly visible after 30 minutes in BAC1.2F5 cells (Fig. 7.6.5., arrows). It was also apparent that M-CSF induced membrane ruffling was not as pronounced in the presence of LY294002.

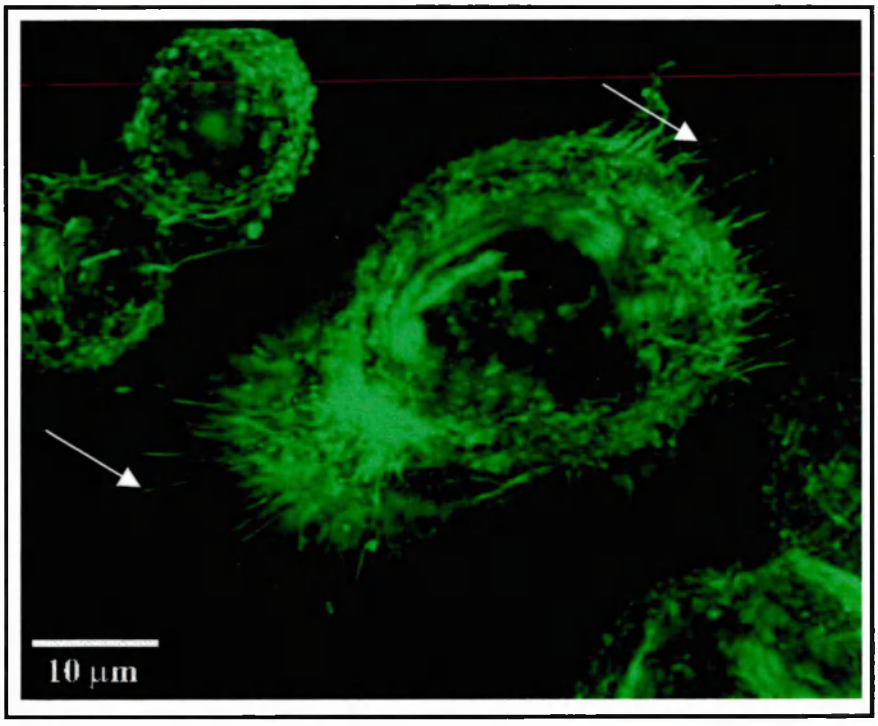


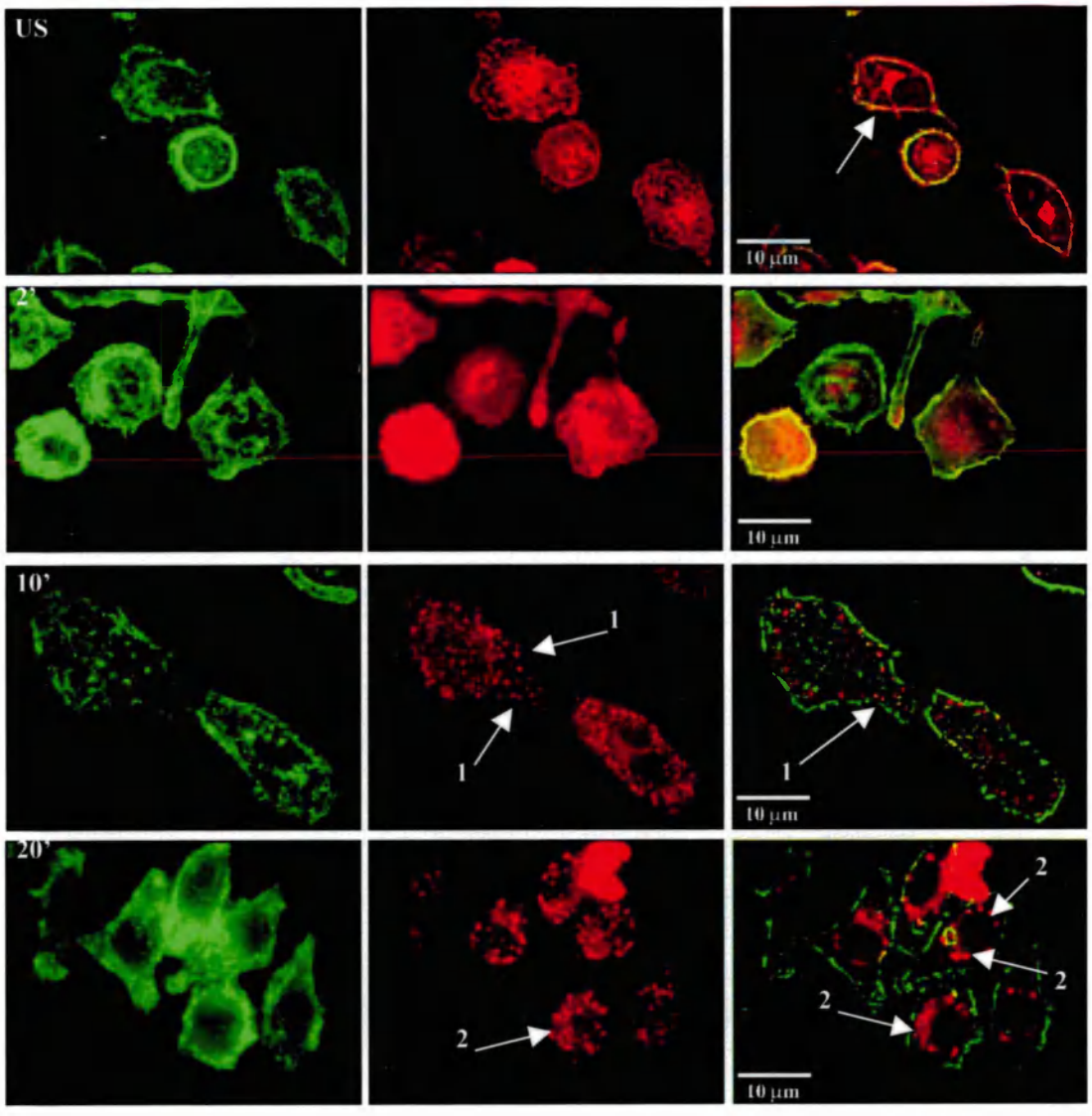
Fig. 7.6.5. Fluorescent micrograph of BAC1.2F5 cells stained for polymerised actin. BAC1.2F5 cells were cultured in the absence of M-CSF for 24 hours before incubation with 10μM LY294002 for 15 minutes, followed by stimulation with 50ng ml^{-1} M-CSF for 30 minutes. Cells were then fixed, permeabilised and stained with a phalloidin-FITC conjugate (Sigma). Images were digitally captured and analysed with OpenLab 1.7.8.. Bar indicates 10μm.

7.7.0. The effect of LY294002 on the subcellular localisation of c-*fms* in BAC1.2F5 cells

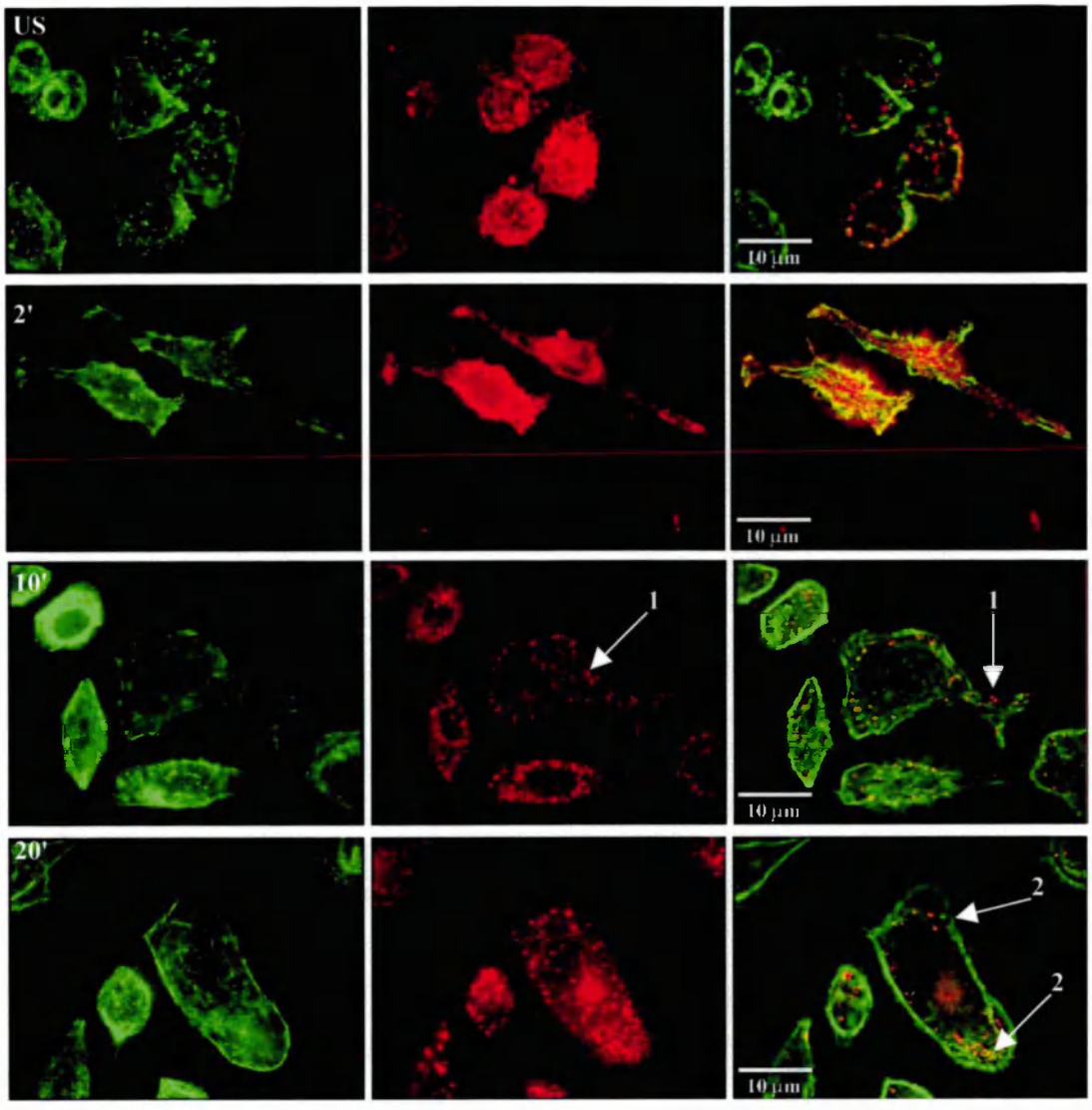
To investigate the effect of inhibition of PI 3-kinase activity on the internalisation, trafficking and degradation of c-*fms* BAC1.2F5 cells were analysed by digital confocal microscopy. The subcellular localisation of c-*fms* was stained immunochemically with an anti-c-*fms* polyclonal antibody whilst F-actin organisation was stained for comparison.

In unstimulated cells c-*fms* was staining was diffuse with both cytosolic and plasma membrane localisation. Examination of a single 1μm section transversely through the field of view by digital confocal fluorescence revealed that the majority of c-*fms* was localised at the plasma membrane (Fig. 7.7.1., left-hand US panel, arrow). After M-CSF stimulation was internalised into small transport vesicles (Fig. 7.7.1., arrow 1), which were visible at a juxtamembrane location after 10 minutes. The transport vesicles moved towards the nucleus and fused together to form larger, macrovesicles, 20 minutes after M-CSF stimulation (Fig. 7.7.1., arrow 2). After 30 minutes c-*fms* staining was absent from the macrovesicles clustered around the nucleus (data not shown), similar to Fig. 7.1.4.

Treatment of BAC1.2F5 cells with 10μM LY294002 did not block the early formation of small transport vesicles which were still visible in a juxtamembrane location (Fig. 7.7.2., arrow 1). However, after 20 minutes, the small vesicles were still present at the plasma membrane and had not translocated to the perinucleus nor had they fused to form large macrovesicles (Fig. 7.7.2., arrow 2).



7.7.1. Fluorescent micrographs of BAC1.2F5 cells stained for polymerised actin and c-*fms*. BAC1.2F5 cells were cultured without M-CSF for 24 hours before stimulation with 50ng ml^{-1} M-CSF as follows; unstimulated (US), stimulated for 2 minutes (2'), 10 minutes (10') or 30 minutes (30'). Cells were then fixed, permeabilised and stained with a phalloidin-FITC conjugate (Sigma) and with an anti-c-*fms* pAb (Santa Cruz). Images were digitally captured with an OpenLab digital image analysis system. Left hand panels are a reconstruction of 25 composite, 0.5μm sections of F-actin staining after software deconvolution. The middle panels are a reconstruction of 25 composite, 0.5μm sections of the same field of view stained for c-*fms*. The right hand panels are a merged composite of two, single 1μm sections from the F-actin and c-*fms* composites. Bars indicates 10μm.



7.7.2. Fluorescent micrographs of BAC1.2F5 cells stained for polymerised actin and c-*fms*. BAC1.2F5 cells were cultured without M-CSF for 24 hours before incubation with 10µM LY294002 before stimulation with 50ng ml^{-1} M-CSF as follows; unstimulated (US), stimulated for 2 minutes (2'), 10 minutes (10') or 30 minutes (30'). Cells were then fixed, permeabilised and stained with a phalloidin-FITC conjugate (Sigma) and with an anti-c-*fms* pAb (Santa Cruz). Images were digitally captured with an OpenLab digital image analysis system. Left hand panels are a reconstruction of 25 composite, 0.5µm sections of F-actin staining after software deconvolution. The middle panels are a reconstruction of 25 composite, 0.5µm sections of the same field of view stained for c-*fms*. The right hand panels are a merged composite of two, single 1µm sections from the F-actin and c-*fms* composites. Bars indicates 10µm.

7.8.0. Discussion

The rapid effects of M-CSF on BAC1.2F5 cells has been extensively characterised with respect to cell morphology and the actin cytoskeleton (Allen et al., 1997; Boocock et al., 1989). It has also been demonstrated previously that upon M-CSF stimulation BAC1.2F5 cells form internalised vesicles which stain for M-CSF (Boocock et al., 1989). Studies by Boocock *et al* have demonstrated that M-CSF is rapidly internalised and trafficked to a perinuclear location (Boocock et al., 1989). However c-*fms* has not been investigated and it has been assumed that it internalises in a manner similar to M-CSF. This chapter formally investigated the internalisation of c-*fms* using indirect fluoroscence in BAC1.2F5 cells. In unstimulated cells c-*fms* is localised to the cytosol, but predominantly the plasma membrane (Fig. 7.1.1.). This is enhanced by incubating cells at 4°C for 30 minutes, a process that slows down receptor internalisation (Fig. 7.1.5.). Stimulation with M-CSF at 37°C results in strong plasma membrane staining in conjunction with small punctate staining around the cell periphery and in the lamellar processes which have remained throughout quiescence (Fig. 7.1.2.). After 10-20 minutes there is extensive staining of internalised vesicles which tend to be larger than the early punctate staining and are probably the products of endosomal fusion (Figs. 7.1.3. and 7.1.4.) (Boocock et al., 1989). These large vesicles progress from the plasma membrane along the lamellar processes towards the cell body, aggregating around the nucleus in a similar pattern to that previously published for M-CSF trafficking (Boocock et al., 1989). After 30 minutes the majority of c-*fms* staining is absent, which correlates with the time-course of c-*fms* degradation observed by western blot analysis in chapter 4. Closer examination of internalised c-*fms* confirmed that c-*fms* co-localised with phase-light endocytic vesicles, which form rapidly (10 minutes) and migrate towards the nucleus

of the cell (Fig. 7.3.1.). These data suggest that c-*fms*, once internalised, is trafficked with its ligand to lysosomal compartments for degradation and does not appear to be recycled back to the plasma membrane.

Upon stimulation of BAC1.2F5 cells with M-CSF, the receptor and various cytosolic proteins are phosphorylated on tyrosine. Analysis of tyrosine phosphorylation by indirect fluorescence has demonstrated that there is a basal level of tyrosine phosphorylation which is found at the plasma membrane and throughout the cytosol (Fig. 7.4.1.). Stimulation at 4°C results in increased levels of tyrosine phosphorylation with the majority being localised to the plasma membrane and very little, if any, present in the cytosol (Fig. 7.4.1.). Recruitment of cytosolic proteins to a plasma membrane is required for their tyrosine phosphorylation and 4°C incubation prevents relocalisation of tyrosine phosphorylated proteins to the cytosol (Fig. 7.4.1.). In addition, to a localisation of tyrosine phosphorylation at the plasma membrane, there is also a recruitment of the p85α and p110α subunits of PI 3-kinase to the plasma membrane (Figs. 7.5.1. and 7.5.2.). These data indicate that M-CSF stimulation actively recruits PI 3-kinase activity to the plasma membrane and therefore into close proximity to substrate and various downstream mediators.

Re-arrangement of the actin cytoskeleton has been extensively investigated, particularly with respect to the rho family of GTPases. However, only recently have the functions of these proteins been studied in macrophages, where the cytoskeleton is critical for motility and cell morphology. It has been reported that M-CSF stimulates the rapid reorganisation of the actin cytoskeleton in BAC1.2F5 cells (Allen et al., 1997; Boocock et al., 1989). This is characterised by the early formation of actin-rich

membrane ruffles in stimulated cells and fine actin cables traversing the interior of the cell (Allen et al., 1997). Formation of membrane ruffles and lamellapodia are regulated by rac which in turn regulates rho-mediated actin cable formation (Allen et al., 1997). M-CSF-stimulated formation of actin-rich filopodia is regulated by cdc42 which has also been shown to inhibit rac and rho activity (Allen et al., 1997). M-CSF-induced rearrangement of the actin cytoskeleton was confirmed by staining cells with FITC-labelled phalloidin and the formation of filopodia, membrane ruffles and fine actin cabling was observed shortly after M-CSF stimulation of BAC1.2F5 cells (Fig. 7.6.1.). High magnification microscopy demonstrated the presence of actin-rich filopodia (Fig. 7.6.2., panel A) following M-CSF stimulation accompanied by formation of actin-rich membrane folds along the dorsal surface of BAC1.2F5 cell (Fig. 7.6.2., panel B). The formation of fine actin cables was also visualised by high magnification microscopy and were characterised as forming organised parallel orientated filaments along the cell axis in polarised cells (Fig. 6.6.3., panel A) and as parallel concentric rings in cells with a more rounded morphology (not shown).

The most striking feature of BAC1.2F5 cells treated with the PI 3-kinase inhibitor, LY294002 was the formation of retraction fibres in the majority of cells in normal culture (Fig. 7.6.4., panel N) and in M-CSF stimulated cells (Fig. 7.6.4., panels N, US and 10'). At higher magnification it is apparent that these retraction fibres are extensive, averaging 10-15μm in length (Fig. 7.6.5., arrows). The formation of actin-rich membrane ruffles was not completely inhibited by treatment with LY294002, however fewer membrane ruffles were observed in BAC1.2F5 cells (Fig. 7.6.4., panel 2'). In addition the formation of actin cables within the cytosol was also inhibited by LY294002 and were still not present up to 30 minutes following M-CSF stimulation

(Fig. 7.6.4., panel 30'). Thus PI 3-kinase activity is required for the M-CSF-stimulated, rho-mediated formation of actin cables within the cell and inhibition of PI 3-kinase activity also partially inhibits rac-mediated membrane ruffle formation. PI 3-kinase is necessary for the activation of rac downstream of the PDGFr (Bokoch et al., 1996; Parker, 1995), and the results presented here indicate that PI 3-kinase activity is required for the activation of rac and rho following M-CSF stimulation of BAC1.2F5 macrophages.

The effect of PI 3-kinase inhibition on the internalisation and trafficking of activated c-*fms* was also investigated using LY294002. Under normal conditions, M-CSF stimulates the rapid internalisation of the ligand receptor complex via the formation of small cytosolic vesicles, present at the plasma membrane 10 minutes after stimulation (Fig. 7.7.1., panel 10', arrow 1). After another 10 minutes the small internalised vesicles have migrated to a peri-nuclear location and have fused to form large macrovesicles (Fig. 7.7.1., panel 20', arrow 2).

Pre-incubation of BAC1.2F5 cells with 10μM LY294002 prior to M-CSF stimulation did not slow or prevent receptor internalisation into the juxtamembrane vesicles which were present by 10' minutes (Fig. 7.7.2., panel 10', arrow 1). However, LY294002 prevented the transport of these vesicles from their juxtamembrane location to a peri-nuclear location, and as a result, vesicle fusion did not occur either (Fig. 7.7.2., panel 20', arrow 2). Therefore for c-*fms*, PI 3-kinase activity is not required during the formation of receptor mediated endocytic vesicles, but is required for the correct trafficking through the cytosol to a juxtanuclear location where they fuse to form macrovesicles and c-*fms* is degraded. The correct trafficking of c-*fms* may rely on the

production of $PI_{3,4,5}P_3$ or one of its degradation products or through the activation of other proteins that control the cellular concentrations of other phospholipids, such as $PI_{4,5}P_2$.

CHAPTER 8

Generation of a human c-*fms* cDNA expression construct, with C-terminal *myc*/His epitope tags, and mutational analysis of key residues within the kinase insert region.

8.0.0. Introduction

The use of PI 3-kinase inhibitors such as wortmannin and LY294002 has made it possible to investigate how PI 3-kinase activity, in general, is involved in c-*fms* signalling. Recent reports have suggested wortmannin can inhibit non-PI 3-kinase dependent signalling pathways, albeit at higher concentrations than were used in this study (Cross et al., 1995). In addition wortmannin and LY294002 are not specific for individual PI 3-kinase family members and it has been demonstrated that wortmannin can inhibit p110α, p110β, p110δ and p110γ in the low nanomolar ranges (Arcaro and Wymann, 1993; Koh et al., 1998; Wymann et al., 1996; Zhang et al., 1998). Therefore, although these inhibitors were capable of defining the M-CSF-mediated responses in BAC1.2F5 cells, they are unable to determine what specific PI 3-kinase isoform activity is stimulated or whether this activation occurs via a direct or indirect mechanism. Therefore the investigation into the recruitment of PI 3-kinase to the c-*fms* signalling complex needs a completely new approach and the method of choice was mutational analysis of the M-CSF receptor.

Recruitment of PI 3-kinase activity to the M-CSF receptor proceeds via binding of SH2 domains in the p85 subunit to specific phosphotyrosine motifs (pYxxM) (Cantley *et al.*, 1991). M-CSF stimulates PI 3-kinase activity in murine bone marrow derived macrophages maximally after ten minutes treatment (Yusoff *et al.*, 1994). This is comparable to the results obtained in BAC1.2F5 cells (Chapter 4, Fig. 4.5.2). In BAC1.2F5 cells, M-CSF stimulates tyrosine phosphorylation of p85α and its stable association with c-*fms* and other cytosolic proteins including p120, p95 and p55-60 (Kanagasundaram *et al.*, 1996). However, only a minor fraction of total cellular PI 3-kinase activity is associated with c-*fms* immunoprecipitates (Kanagasundaram *et al.*, 1996). Therefore association of only a small proportion of the cellular pool of PI 3-

kinase with c-*fms* has important implications for analysis of the activation of PI 3-kinase and its detection when bound to activated c-*fms*.

The p85 subunit of PI 3-kinase contains two SH2 domains and its interactions with other proteins is complex, for example its association with IRS-1 requires occupancy of both SH2 domains for full PI 3-kinase activation (Rordorf-Nikolic et al., 1995). However, only the C-terminal SH2 domain mediates association with the PDGFαr through Tyr^{751} which leaves the N-terminal SH2 domain unoccupied and capable of binding to other moieties (Kavanaugh et al., 1992). The binding site for PI 3-kinase on the PDGFβr has been mapped to not one but two residues, Tyr731 and Tyr742, which are both essential for PI 3-kinase activation (Yu et al., 1994). The tyrosine residue required for PI 3-kinase association to phosphorylated c-*fms* has been mapped *in vitro* to Tyr^{721} of the murine receptor (Reedijk *et al.*, 1992). In close proximity to Tyr^{721} is another tyrosine phosphorylation site at Tyr^{706}. The spacing of the these two tyrosine phosphorylation sites (15 amino acids) is similar to that seen in the PDGFβr (11 amino acids) and it is therefore is possible that both p85 SH2 domains are involved in PI 3-kinase association with c-*fms*.

In a yeast two hybrid study an autophosphorylated cytoplasmic domain of c-*fms* was used as bait to screen an expression library and clones containing the SH2 domains of PLCγ2 were shown to interact with Tyr^{721} (Bourette *et al.*, 1997). In c-*fms* expressing FDC-P1 cells, M-CSF stimulated the rapid, transient phosphorylation of PLCγ2 and activation of PLCγ2 was required for activation of the PI 3-kinase pathway (Bourette *et al.*, 1997). It is possible that in FDC-P1 cells the relative levels of PLCγ2 and PI 3-kinase are different to those found in other cell types and this data may be an artefact

of this feature, however it does indicate that it is possible that PLCγ2 and PI 3-kinase can compete for the same binding site on c-*fms* or alternatively, PLCγ2 can provide an alternative mechanism for PI 3-kinase activation.

A requirement of PI 3-kinase activity during c-*fms* mediated responses has not been extensively studied in macrophages, although it is implicated in such functions as survival, proliferation, vesicle transport and changes in cell morphology. The reason for this is because almost all analysis of c-*fms* signalling has been carried out in exogenous expression studies in cell lines such as fibroblasts that are easily transfected and produce high levels of exogenous expression. However studies in fibroblasts have produced conflicting data (Shurtleff *et al.*, 1990; Reedijk *et al.*, 1992; van der Geer and Hunter, 1993). For instance, in Rat-2, or NIH 3T3 fibroblasts, expression of mutated human c-*fms* without the capacity to bind PI 3-kinase via Tyr^{723} were unable to activate PI 3-kinase with a concomitant loss in cell proliferation (Reedijk et al., 1992; van der Geer and Hunter, 1993). In a macrophage based study, PI 3-kinase inhibitors blocked endocytosis by preventing vesicle trafficking, rather than vesicle formation (Araki et al., 1996). Conversely, in FDC-P1 cells transfected with a KI domain c-*fms* mutant, cell proliferation was still stimulated by M-CSF at levels similar to wild type c-*fms* (Kanagasundaram et al., 1996). Ratification of the conflicting data on the involvement of PI 3-kinase in c-*fms* signalling was the overall aim of this thesis and therefore the requirement of PI 3-kinase activity in cellular responses such as apoptosis, vesicle transport and cytoskeletal organisation, which has been clearly demonstrated in the preceding chapters can only be examined in more detail by mutational analysis of c-*fms* at the residues critical for PI 3-kinase association.

The main aim of this chapter was the production of two human c-*fms* expression constructs, one representing the full length c-*fms* cDNA and the second representing the same cDNA but with the addition of sequence at the C-terminus corresponding to two epitope tags. The epitope tagged c-*fms* construct would then be used as a template for mutagenesis of those residues shown to be required or possibly involved in the direct association of PI 3-kinase and its activation. The two tyrosine phosphorylation sites at Tyr^{708} and Tyr^{723} were mutated singly or in combination to phenylalanine. It was intended that these receptor constructs would be expressed in BAC1.2F5 cells or alternative cell lines to investigate further the involvement of PI 3-kinase in c-*fms* signalling. The two epitope tags were included at the C-terminus to facilitate downstream identification of expressed protein and to aid affinity purification of c-*fms*, and co-purification of PI 3-kinase and other associated proteins in M-CSF activated cells.

8.1.0. Cloning of human c-*fms* into a mammalian expression vector (I)

A construct containing the full-length human c-*fms* gene (pZipSV(X)/c-*fms*) was kindly provided by C. Sherr (Howard Hughes Medical Institute, Memphis, TN). This was used as a template for construction of the c-*fms* expression constructs and its insertion into pcDNA3.1-based mammalian expression vectors (Invitrogen, Appendix C).

Cloning of a full length c-*fms* gene by polymerase chain reaction (PCR) (Mullis and Faloona, 1987) alone may have introduced unwanted errors into the cloned sequence, therefore the initial strategy involved liberation by restriction enzyme digestion the 5' end of c-*fms* as a 2.1kbp fragment (5' RF). Two unique restriction enzyme sites, Eco RI and Xho I, conveniently situated upstream and downstream of the start codon, facilitated this approach. Cloning of the remainder of c-*fms* required cloning both an intact (3'PCR1) and a disrupted 3'-end (3'PCR2) by PCR with gene-specific primers. The 5' primer encompassed an internal c-*fms* Xho I site and facilitated the cloning together of each c-*fms* fragment. The homologous, 3'PCR1 product was to be amplified with a homologous primer containing a Hind III site, downstream of the c-*fms* stop codon. The disrupted 3'-end PCR product was to be amplified with an alternate 3' primer which encompassed the sequence containing the c-*fms* stop codon. This alternate primer contained a unique Hind III restriction enzyme site in place of the stop codon to facilitate downstream subcloning into a pcDNA3.1 based vector. The two c-*fms* fragments, 5'RF and 3'PCR (1 and 2) would then be subcloned, in tandem, into the relevent expression vector linearised by restriction enzyme digestion with Eco RI and Hind III. Fig. 8.1.0. illustrates the cloning strategy employed.

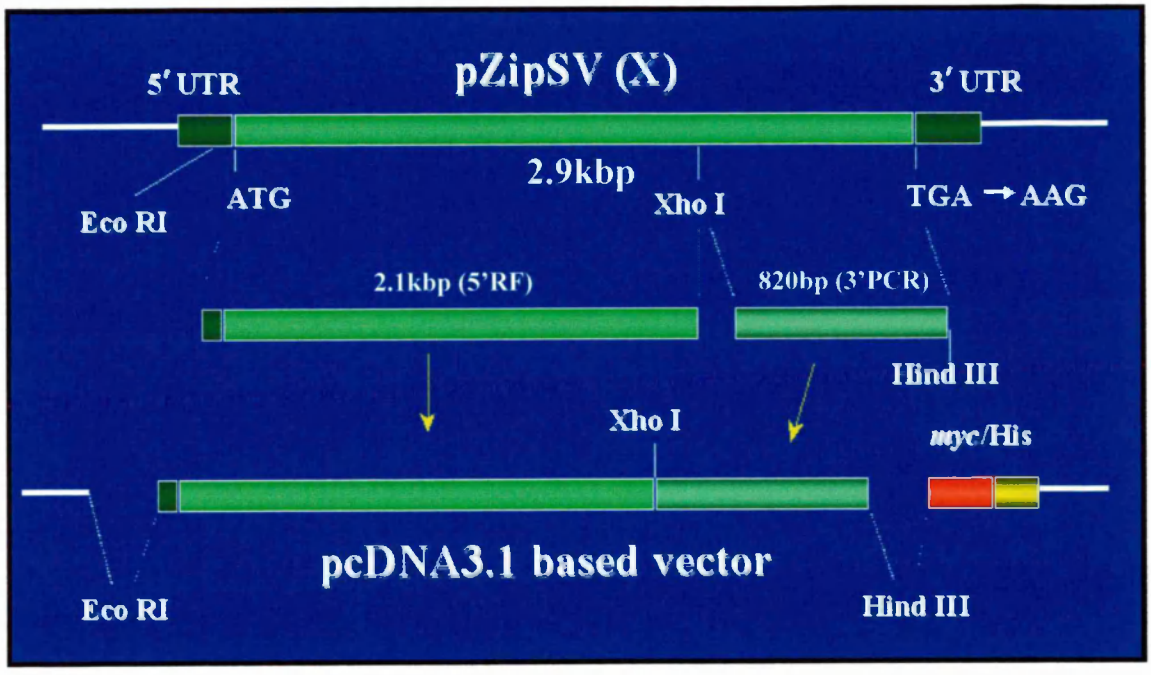


Fig. 8.1.0. Schematic of the human c-*fms* cloning strategy for construction of an epitope tagged mammalian expression construct.

8.1.1. PCR amplification of the 3' end of human c-*fms* (I)

Initially production of 3'PCR1 and 3'PCR2 was attempted by PCR amplification of the 3' end of c-*fms* with a combination of the primers 5' Xho I (AF-1) and 3'Hom (AR-2) for 3'PCR1 and 5' Xho I (AF-1) and 3' Hind III (AR-1) for 3'PCR2 (Appendix B) using pZipSV(X)/c-*fms* as a template. Repeated attempts to amplify up either product failed to yield any products. Reaction conditions were altered including; [Mg^{2+}], primer concentrations and thermal cycling parameters in an attempt to obtain product but without success (Data not shown). As an alternative to amplification from cDNA, RNA was prepared from U937 cells and used as an alternative template for amplification by reverse transcription PCR (RT-PCR). The two primers, AF-1 and AR-1 corresponding to 3'PCR2 amplified a product of 650 bp. from U937 RNA (Fig. 8.1.1.). Although smaller than expected the 650 bp product had been specifically amplified and it could represent a differentially spliced version of c-*fms*. The 650bp product was cloned into pCR2.1 vector and analysed by restriction digest prior to sequence analysis.

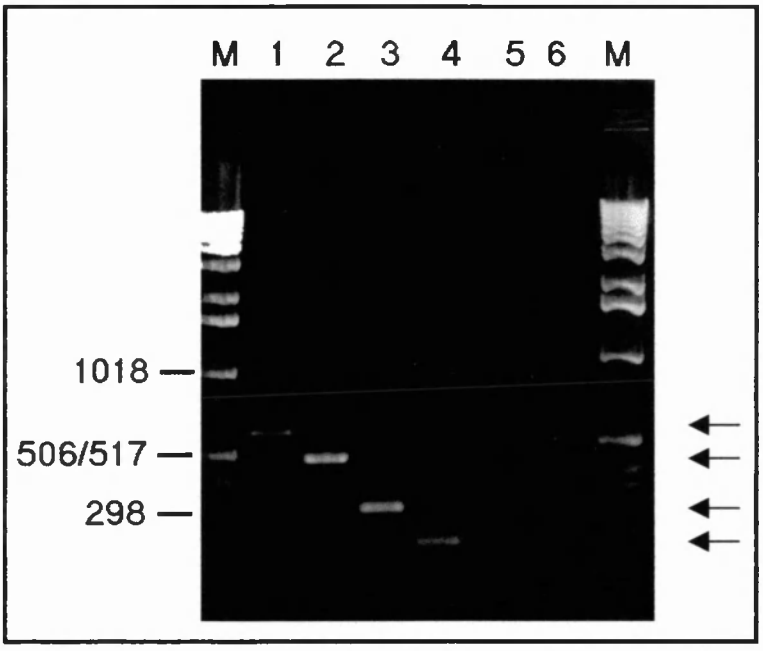


Fig. 8.1.1. Analysis by 1% gel electrophoresis of the products of the amplification of the 3' end of human C-*fms* from U937 RNA using primers AF-1 and AR-1. Lane 1 represents RT-PCR amplification from a U937 RNA template (primers at 0.5μM). Lane 2 represents bacteriophage lambda positive control (500 bp). Lane 3 represents pAW109 RT-PCR positive control (308 bp). Lane 4 represents a second RT-PCR positive control amplification of the SH3 domain of Lyn tyrosine kinase (≈200 bp). Lanes 5 and 6 represents a PCR and RT-PCR no template controls, respectively. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

Unexpectedly during cloning a 1050 bp insert product was liberated from positive clones (Fig. 8.1.2.). Both inserts were sequenced using the dye-deoxy terminator cycle method. By comparison to the published c-*fms* sequence, the 650 bp product was found, overall, to be less than 35% homologous and the 1050 bp product was only 30% homologous to c-*fms*. A Blastn sequence similarity search (Altschul et al., 1997) indicated that the 650bp and 1050bp products had low homology to various genes from multiple organisms including the human and murine c-*fms* but these homologies were less than 35%. As it was a possibility that c-*fms* mRNA was not present in abundance in the U937 RNA preparation, the number of PCR cycles had been extended. This would account for the increased likelihood of obtaining non-

specific amplification products suggesting that the 650bp and 1050bp RT-PCR products were more likely to be PCR artefacts.

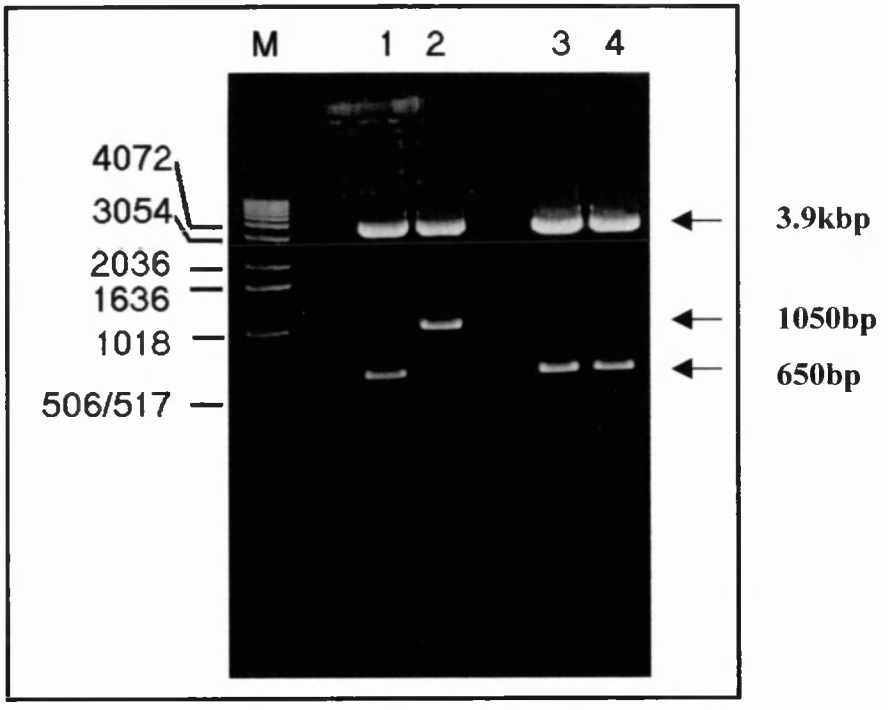


Fig. 8.1.2. Analysis by 1% agarose gel electrophoresis analysis of Eco R1 digest analysis of putative clones containing the 650 bp PCR fragment cloned into pCR2.1. Plasmid DNA was isolated from positive clones and digested with Xho I and Hind III before resolving by agarose gel electrophoresis. Lanes 1, 3 and 4 are 650bp clones. Lane 2 is a 1050bp clone. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

8.2.1. Amplification of the 3' end of c-*fms* by PCR (II)

A further attempt was made to amplify the 3' end of c-*fms*. In an effort to circumvent potential problems with primer accessibility due to problems with secondary structure, pZipSV(X)/c-*fms* was first linearised by enzymatic digestion with Eco RI. Original supercoiled plasmid DNA was also used as a template for comparison. In addition, a new primer directed to the 3'-end was synthesised to prevent problems associated with reduced annealing efficiency of the AR-2 PCR primer, which had a restriction site present downstream of the stop codon for cloning purposes. This new primer, AR-3 was designed completely homologous to c-*fms* encompassing the stop codon of c-*fms*.

Fig. 8.2.1. shows products obtained from various primer and template combinations. Using both primer combinations the two 3' PCR products were amplified successfully from cDNA template. There was no apparent difference between the products obtained from linear or supercoiled template DNA.

AF-1	+	+	+	+
AR-1	-	-	+	+
AR-3	+	+	-	-
Linear template	+	-	+	-
Supercoiled template	-	+	-	+

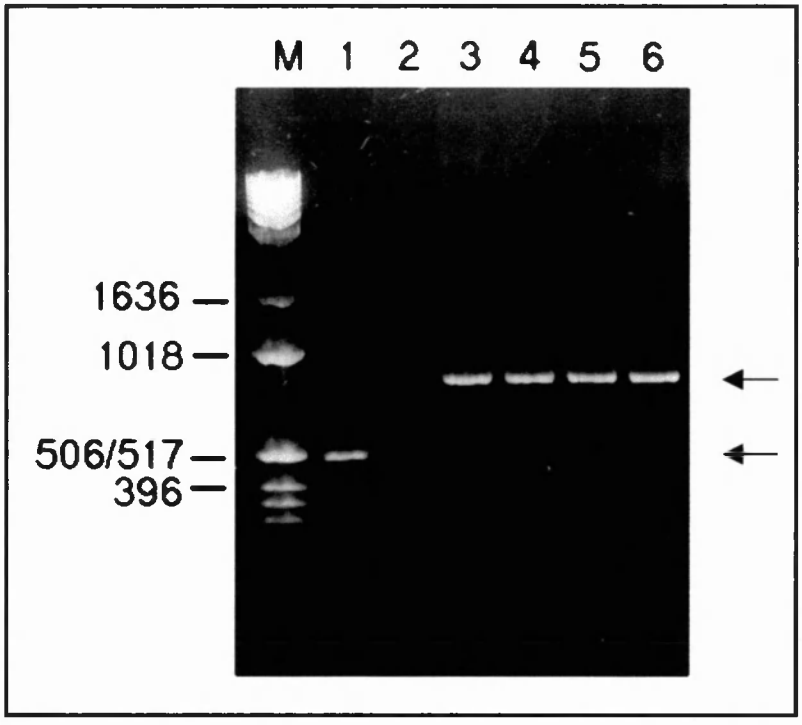


Fig. 8.2.1. Analysis by gel electrophoresis products of PCR amplification of a fragment of the 3' end of c-*fms* from cDNA template. Lane 1 represents bacteriophage lambda positive PCR control (500 bp), lane 2 represents a no template, negative control. Lanes 3, 4, 5 and 6 represent amplification products. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

Each product was cloned into pCR2.1, transformed into *E. coli* and plasmid DNA was prepared. At this point only clones from E. coli transformed with pCR2.1 containing the 3'PCR2 amplification product were chosen for subsequent analysis by restriction enzyme digestion and sequencing. Xho I/Hind III restriction enzyme analysis of five clones representing 3'PCR2 (lanes 2, 3, 4, 6, and 7, respectively) showed they

contained the correct sized, 820bp insert (Fig. 8.2.2). A representative clone was selected for further analysis by sequencing.

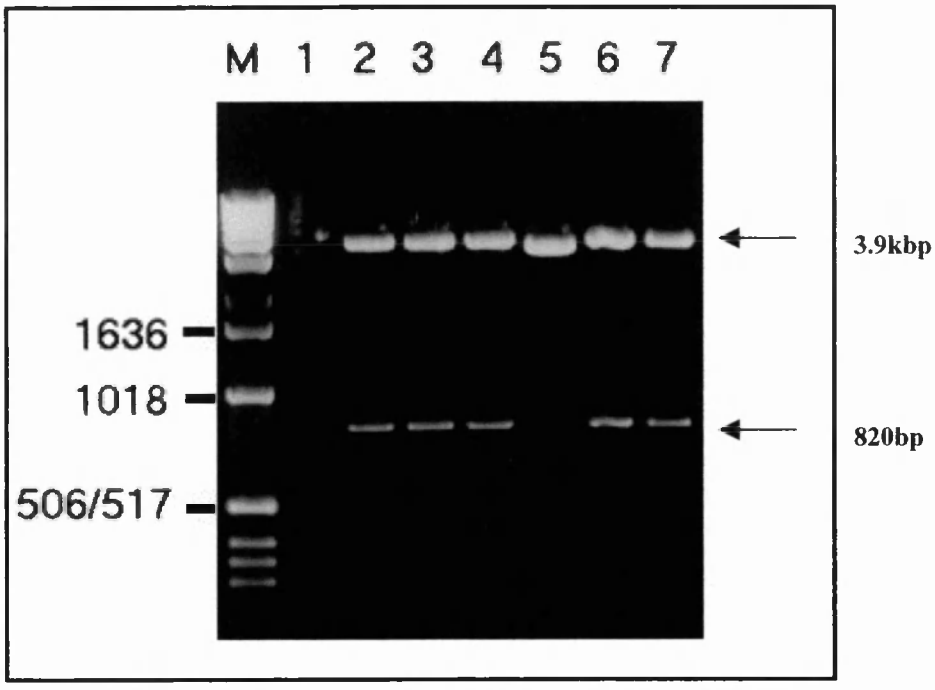


Fig. 8.2.2. Analysis by 1% gel electrophoresis of the products of a Xho I/Hind III restriction digest of pCR2.1(3' c-*fms*). Plasmid DNA of clones isolated from *E. coli* transformed with pCR2.1 containing the 820 bp PCR product from the amplification of the 3' end of c-*fms* were digested with Xho I and Hind III before resolving on an agarose gel. Lanes 2 to 7 contain positive clones. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

8.3.1. Sequence analysis of 3'PCR2 product

Sequence analysis of a clone obtained from section 8.2. demonstrated sequence homology c-*fms* and possessed the required 5' Xho I site and the c-*fms* stop codon mutated to a Hind III (Data not shown).

8.4.1. Liberation of the 5' portion of c-*fms* from pZipSV(X)/c-*fms*

The pZipSV(X)/c-*fms* construct contained a previously engineered Eco RI restriction enzyme site upstream of the start codon, within the 5' untranslated region (UTR) of c-*fms*. However, the exact location of the Eco RI site was not indicated in the published sequence (Accession No. X03663). This Eco RI site was used in conjunction with the Xho I site at 2110bp in the c-*fms* coding region, to liberate a fragment containing a portion of the 5' UTR as well as the first 2110bp of c-*fms* (5'RF) (Fig.8.1.0., 2.1kbp arrow).

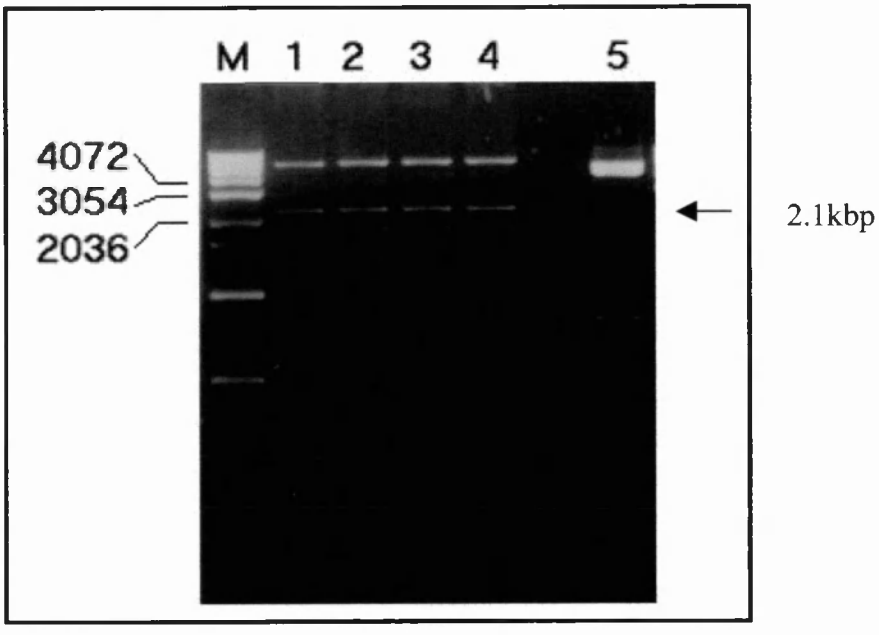


Fig. 8.4.1. Analysis by 1% gel electrophoresis of products of an Eco RI/Xho I restriction enzyme digest of purified plasmid DNA from *E. coli* transformed with pZipSV-(X)/c-*fms*. Lanes 1-4 contain pZipSV-(X)/c-*fms* digested with Eco RI and Xho I. Lane 5 is 3'PCR2 digested with Xho I/Hind III. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

Lanes 1-4 of Fig. 8.5.1. show an Eco RI/Xho I digest of pZipSV(X)/c-*fms* with five visible products including a band of ≈2.1kbp, which corresponds to the fragment of interest (Fig. 8.4.1.).

8.5.1. Cloning of the 5'RF and 3'PCR2 into pcDNA3.1$^{(-)}$*Myc*/His

The pcDNA3.1$^{(-)}$*Myc*/His vector was linearised by digestion with Eco RI /Hind III and gel purified. The 2.1kbp, 5'RF was liberated from pZipSV(X)/c-*fms* by digestion on Eco RI and Xho I (Chapter 8, section 5.1.). The 3' PCR product was liberated from pCR2.1 by digestion with Xho I and Hind III and both inserts were gel purified. The linearised vector, 5'RF and 3'PCR2 were ligated together and ligation products were transformed into *E. coli.* Colonies were selected, plasmid DNA was prepared and analysed by restriction digest with Eco RI and Hind III. Unexpectedly a 1.2kbp and a 1.7kbp product were liberated from pcDNA3.1$^{(-)}$*Myc*/His instead of the expected 2.9kbp (data not shown). The spurious cloning results were possibly due to the difficulty of cloning two fragments simultaneously into the pcDNA3.1 vector. To simplify ligation an alternative cloning strategy was adopted that involved the use of a shuttle vector, which facilitated subcloning each c-*fms* fragment separately prior to subcloning as a single fragment into a pcDNA3.1 based vector.

8.6.1. Cloning of the 5' RF and 3'PCR2 into pGEM 11Zf(+)

As an alternative strategy, the 5' RF and 3' PCR product were cloned into a shuttle vector, pGEM 11Zf(+) (Appendix C). Figure 8.6.1. illustrates this cloning strategy. Since it had proved too difficult to ligate both the 5'RF and 3'PCR into pcDNA3.1$^{(-)}$*myc*/His simultaneously, ligation of each fragment in turn was the next logical choice. However, the orientation and positions of restriction sites in the pcDNA3.1 multiple cloning site (MCS) rendered it impossible to clone each fragment in separately and an alternative strategy was designed that utilised a cDNA cloning vector with a suitable MCS, pGEM11Zf(+). The nature of the pGEM MCS facilitated cloning of the 3'PCR in on Xho I and Hind III enzyme sites to produce pGEM3'PCR (Fig. 8.6.1., A and B). After successful cloning, pGEM3'PCR could then be linearised by digestion on Eco

RI and Xho I (Fig. 8.6.1., C) to facilitate subcloning of the 5'RF fragment into pGEM3'PCR (Fig. 8.6.1., D and E). Positive clones containing both fragments, (pGEM/c-*fms*) could then be digested with Eco RI and Hind III to liberate a fragment containing c-*fms* ready for cloning into a pcDNA3.1 based vector (Fig. 8.6.1., F).

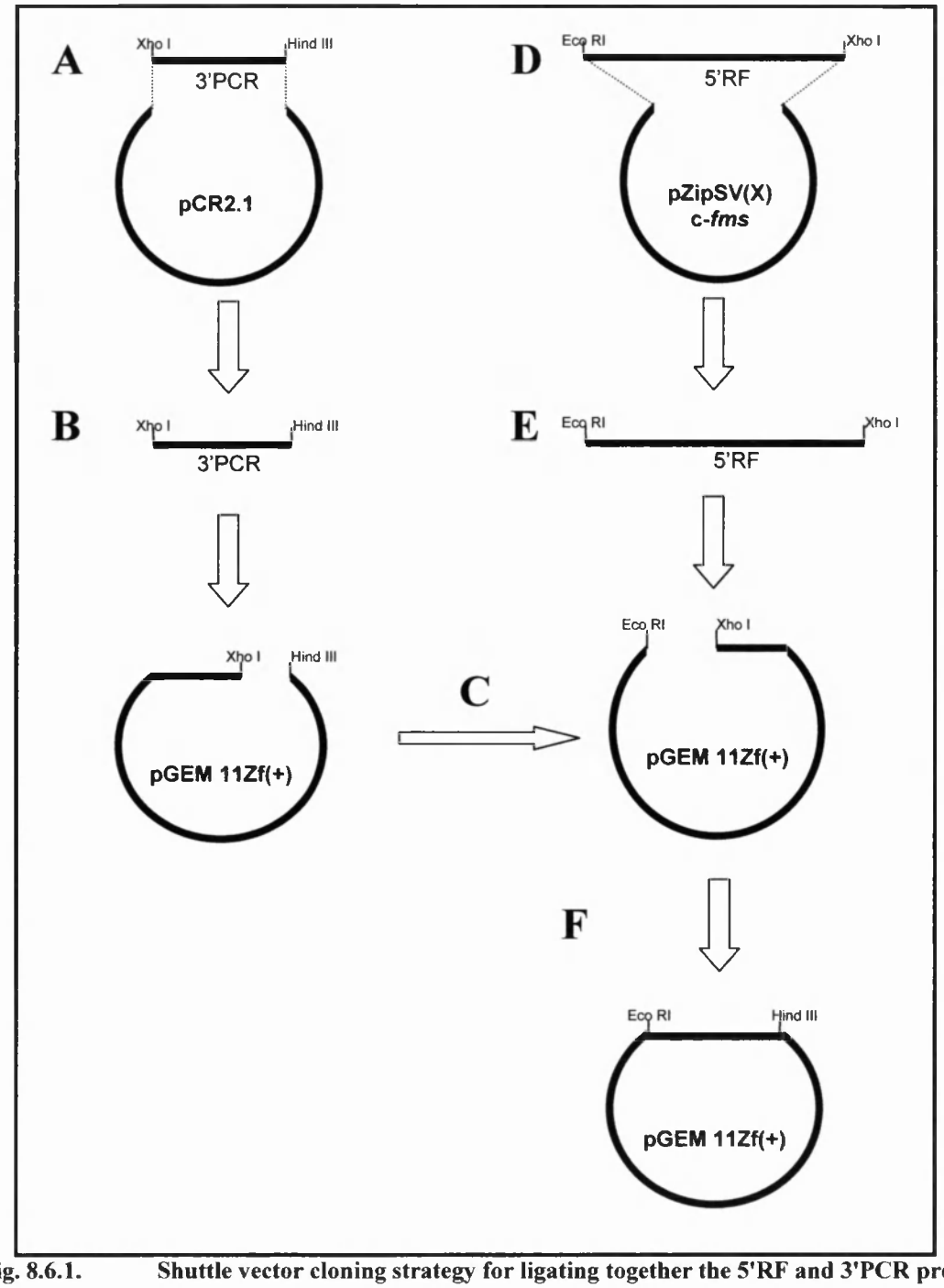


Fig. 8.6.1. Shuttle vector cloning strategy for ligating together the 5'RF and 3'PCR product in a pGEM based cloning vector prior to subcloning into a pcDNA3.1 based vector.

The pGEM 11Zf(+) vector was linearised by digestion with Xho I and Hind III and gel purified before insertion of the 3'PCR (data not shown). Positive pGEM3'PCR clones were selected and analysed by restriction enzyme digestion with Xho I and Hind III (data not shown). A clone containing pGEM3'PCR was linearised with Eco RI and Xho I and the 4.1kbp product was gel purified. The 5'RF fragment was liberated from pCR2.1 and gel purified prior to ligation into the gel purified pGEM3'PCR. Clones containing the ligation product of pGEM3'PCR and 5'RF (pGEM/c-*fms*) were selected for further analysis. Restriction digest analysis of selected clones with Eco RI and Hind III to liberate the full length c-*fms* fragment repeatedly and unexpectedly produced products of 3.0, 1.8 and 1.2kbp, not the predicted 3.2 and 2.9kbp (Data not shown).

More detailed analysis revealed that digestion of the pGEM/c-*fms* with either Eco RI or Xho I produced a single band that migrated at the predicted molecular weight of 6.1kbp for pGEM containing both c-*fms* fragments (Fig. 8.6.2., lanes 1 and 2).

Digestion with Hind III, was also expected to produce a linearised fragment, but repeated analysis produced a spurious bands of ≈1.9kbp and 4.1kbp (Fig. 8.6.2., lane 3). A double digest was carried out with Xho I and Hind III also conflicted with the predicted products of 5.2kbp and 820bp and instead bands of 4.1kbp, 1.1kbp and 820bp were present (Fig. 8.6.2., lane 4).

Taken together these data suggested that another Hind III site was present in the 5' RF. Computer prediction of restriction enzyme sites in the human c-*fms* mRNA originally predicted no Hind III sites within the c-*fms* coding sequence (MacDNAsis v2.6). However a newer search revealed that a Hind III site was indeed present at

1349bp, in the middle of the 5'RF. The presence of this Hind III site explained all the observed problems associated with cloning into both pcDNA3.1$^{(-)}$*myc*/His and pGEM11Zf(+).

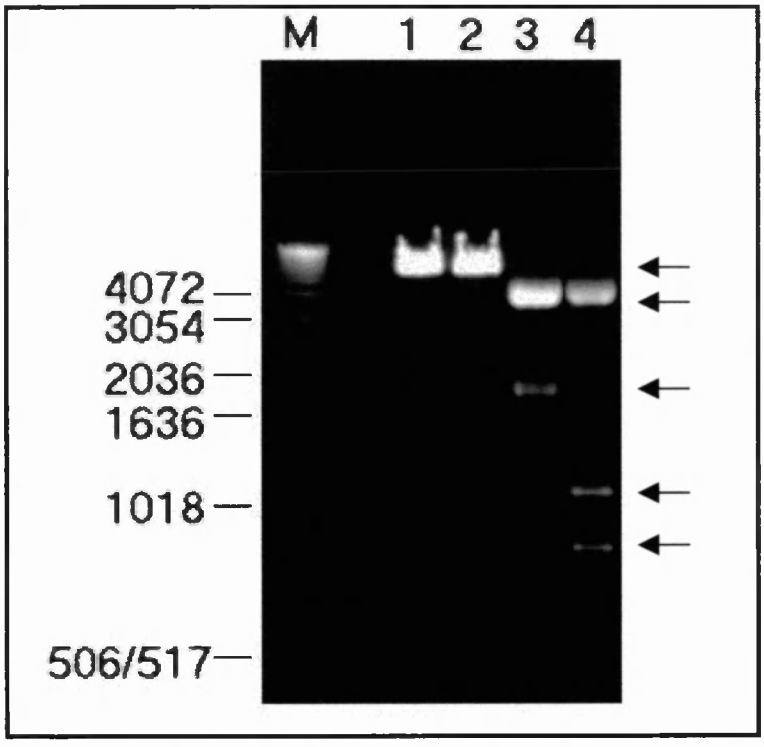


Fig. 8.6.2. Analysis by 1% gel electrophoresis products of diagnostic restriction enzyme digests from pGEM clones. Lanes 1 and 2 are pGEM 11Zf(+)/c-*fms* linearised with Eco RI and Xho I, respectively (6.1kbp). Lane 3 represents Hind III digested pGEM 11Zf(+)/c-*fms* (4.1kbp and 1.9kbp) and lane 4 represents Xho I/Hind III digested pGEM 11Zf(+)/c-*fms* (4.1kbp, 1.1kbp and 820bp.). Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

Unfortunately, because of the orientation of the MCS in the pcDNA3.1$^{(-)}$ vector there were no other available enzyme sites that would have facilitated subcloning of the full length c-*fms* from pGEM into pcDNA3.1. Thus although the full c-*fms* gene was present in pGEM, and appeared to have been cloned successfully, it was impossible continue and subclone into pcDNA3.1$^{(-)}$*myc*/His. Further cloning of c-*fms* into a pcDNA3.1 based vector required another, alternative approach.

8.7.1. Amplification of full-length c-*fms* using a proof-reading DNA polymerase

The availability of molecular biology tools for the reliable amplification of DNA fragments of 3.0kbp and larger made it possible to reconsider amplification of the full length c-*fms* cDNA, an approach that had originally not been chosen because of the high probability of errors that would have resulted in non-sense mutations. Therefore a new approach was designed around using a proof-reading DNA polymerase to amplify a cDNA encompassing the 5' Kozak consensus sequence, and the full c-*fms* coding sequence. Fig. 8.7.1. is a schematic representation of the new cloning strategy. Amplification of the full length c-*fms* coding sequence would allow amplification of a completely homologous c-*fms* construct (WT-1), as well as amplification of a product containing a short linker in place of the stop codon for production of a tagged construct (MT-1). The availability of newer pcDNA3.1 based vectors with a MCS in the positive orientation also allowed subcloning of MT-1 from a pCR2.1 cloning vector into the new, pcDNA3.1$^{(+)}$*myc*/His expression vector using a 3' restriction enzyme site other than Hind III.

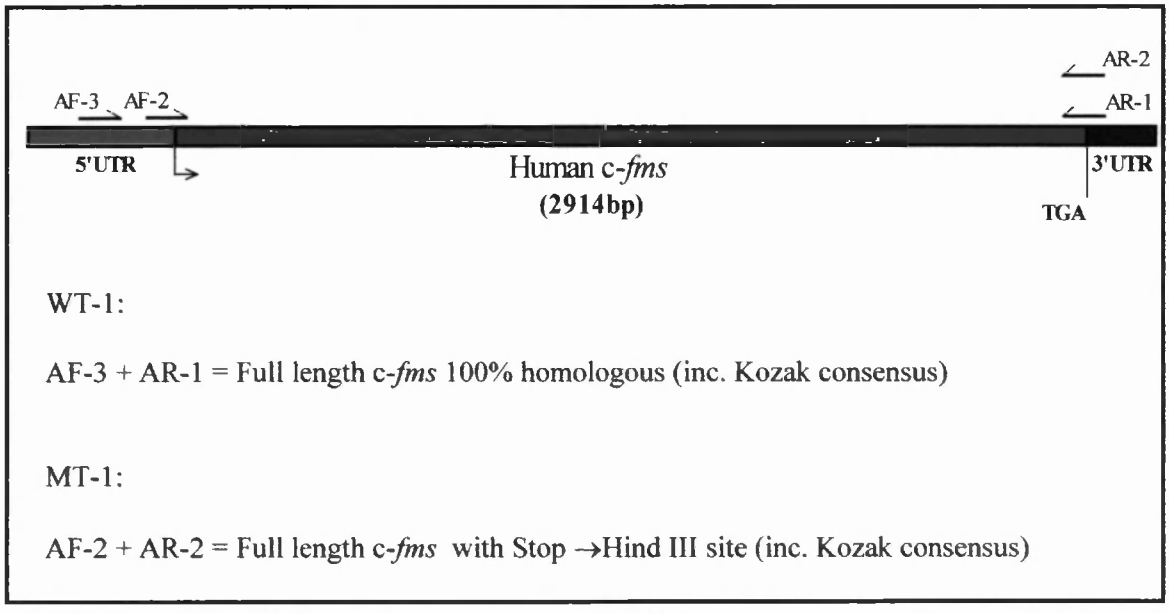


Fig. 8.7.1. Strategy for cloning c-*fms* by PCR amplification using a proof-reading DNA polymerase.

Amplification of the full-length c-*fms* gene from pZipSV(X)/c-*fms* was carried following the Advantage™ KlenTaq (Clontech) PCR methodology (Chapter 3.4.11.). The KlenTaq DNA polymerase mix contains both a standard DNA polymerase as well as a polymerase with proof-reading ability. The proof-reading DNA polymerase facilitates efficient and accurate amplification from cDNA template of products up to 40 kbp.

PCR amplification of c-*fms* was carried out with primer sets AF-3 and AR-1 for amplification of WT-1 and AF-2 and AR-2 for amplification of MT-1 from either U937 cDNA library (Clontech) or cDNA containing pZipSV(X)/c-*fms*. Initial amplification from the U937 cDNA library was unsuccessful, due to the possible low abundance of c-*fms* cDNA. No amplification products were obtained from U937 cDNA corresponding to the predicted molecular weight (Fig. 8.7.2., lanes 1 and 2) for either WT-1 or MT-1. The nature of the U937 cDNA library production may have accounted for a low abundance of c-*fms* cDNA since the library had been prepared by reverse transcription of an oligo-dT primer. The long 3'UTR of c-*fms* may have decreased the likelihood of successful synthesis of cDNA.

However, in contrast, both WT-1 and MT-1 were successfully amplified from pZipSV(X)/c-*fms* (Fig. 8.7.2., lanes 3 and 4, respectively). Both 2.9kbp PCR products were gel purified for subsequent ligation into pCR2.1.

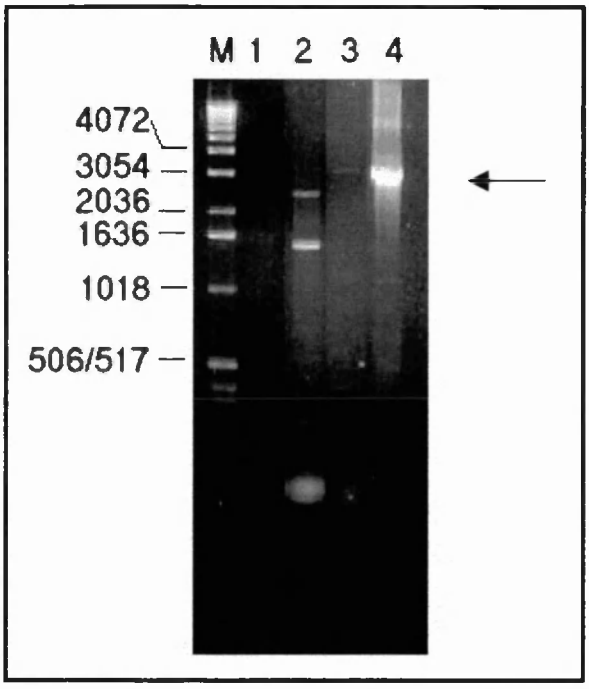


Fig. 8.7.2. Analysis by 1% gel electrophoresis of products from the amplification of full length c-*fms* by PCR from U937 cDNA library and pZipSV(X)/c-*fms*. Lanes 1 and 2 represent amplification products from U937 cDNA. Lanes 3 and 4 represent amplification products from plasmid DNA template. Lanes 1 and 3 are products from primers AF-2 and AR-2. Lanes 2 and 4 are products of primers AF-3 and AR-1. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

8.7.2. Cloning and analysis of products obtained from PCR of full length c-*fms*

The 100% Homologous (WT-1) and 100% Engineered (MT-1) products obtained from pZipSV(X)/c-*fms* were cloned into pCR2.1 and transformed into *E.coli*. Representative clones of WT-1 and MT-1 were then sequenced (data not shown). Sequence analysis confirmed that the WT-1 clones were >99% homologous to c-*fms* and the MT-1 clones were >98% homologous to c-*fms* only differing, as expected, at the engineered, 3'-site (Data not shown). The two c-*fms* clones were then ready for cloning into pcDNA based expression vectors.

8.8.1. Sub-cloning of WT-1 into pcDNA3.1$^{(-)}$

The WT-1 clone was liberated from pCR2.1 by restriction enzyme digestion with Eco RI, cutting at two Eco RI sites in the MCS of pCR2.1, one upstream the other downstream of c-*fms*, before gel purification. In addition, the pcDNA3.1$^{(-)}$ vector had been linearised with Eco RI and treated with calf intestinal alkaline phosphatase (CIAP, Promega) to remove 5'-phosphate groups and to minimise vector recircularisation.

Clones containing WT-1 ligated into pcDNA3.1$^{(-)}$ were analysed by colony PCR (Chapter 3.4.11) with their amplification primers (Data not shown) and six positive clones were selected for further analysis by restriction enzyme digestion (Fig. 8.8.1.).

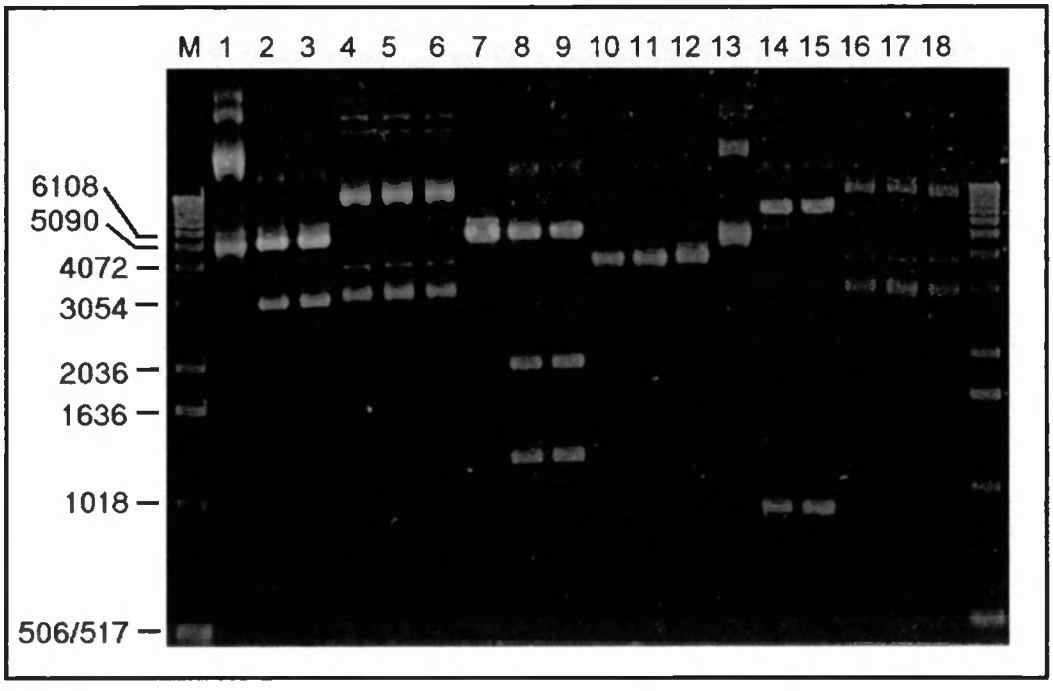


Fig. 8.8.1. Restriction enzyme analysis of positive clones containing WT-1. Six clones were analysed by restriction enzyme digestion with Eco RI (lanes1-6), Eco RI and Hind III (lanes 7-12) and Xho I (lanes 13-18). The predicted products of WT-1 digestion were 5.4kbp and 2.9kbp for Eco RI, 5.3kbp, 1.9kbp and 1.1kbp for the Eco RI/Hind III digest and 7.5kbp and 820bp for the Xho I digest. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

Two positive clones were identified from restriction enzyme digestion. Digestion with Eco RI liberated two bands of 5.4kbp and 2.9kbp representing the vector back-bone and the c-*fms* insert (Fig. 8.8.1., lanes 2 and 3). A double digest with Eco RI and Hind III liberated three bands of 5.3kbp, 1.9kbp and1.1kbp, representing the vector back-bone, an Eco RI/Hind III fragment containing the 5' of c-*fms* and a Hind III fragment containing the rest of c-*fms* (Fig. 8.8.1., lanes 8 and 9). Digestion with Xho I yielded two bands of 7.5kbp and 820bp corresponding to the Xho I site within c-*fms* and the 3' Xho I site in the vector MCS (Fig. 8.8.1., lanes 14 and 15). Restriction enzyme analysis also suggested that both these clones were in the correct orientation in pcDNA3.1$^{(-)}$. Positive clones revealed by restriction digest were sequenced (Appendix D) and were confirmed to contain human c-*fms*, in the correct orientation as indicated by enzymatic digestion.

8.8.2. Sub-cloning of MT-1 into pcDNA3.1$^{(+)}$-*myc*/His

To facilitate cloning of MT-1 into a pcDNA3.1 based vector containing a C-terminal *myc*/His tag, two restriction enzyme sites were chosen for cloning purposes that were common to the MCS of pCR2.1 and pcDNA3.1 vectors in the positive orientation (+). A recently available variant of the vector originally chosen for pcDNA3.1$^{(+)}$-*myc*/His, facilitated sub-cloning from the pCR2.1 vector on BamH I/Not I, two sites unique to the MCS and not present in c-*fms*. Clones containing MT-1 in pCR2.1 digested with BamH I and Not I restriction enzymes and the 2.9kbp band liberated was gel purified. The gel purified, MT-1 was ligated, into pcDNA3.1$^{(+)}$ *Myc*/His (Frame C) and transformed into *E. coli*. Positive clones were screened by colony PCR with AF-2 and AR-2 and clones containing the correct sized insert were also analysed by restriction enzyme digestion (Fig. 8.8.2.).

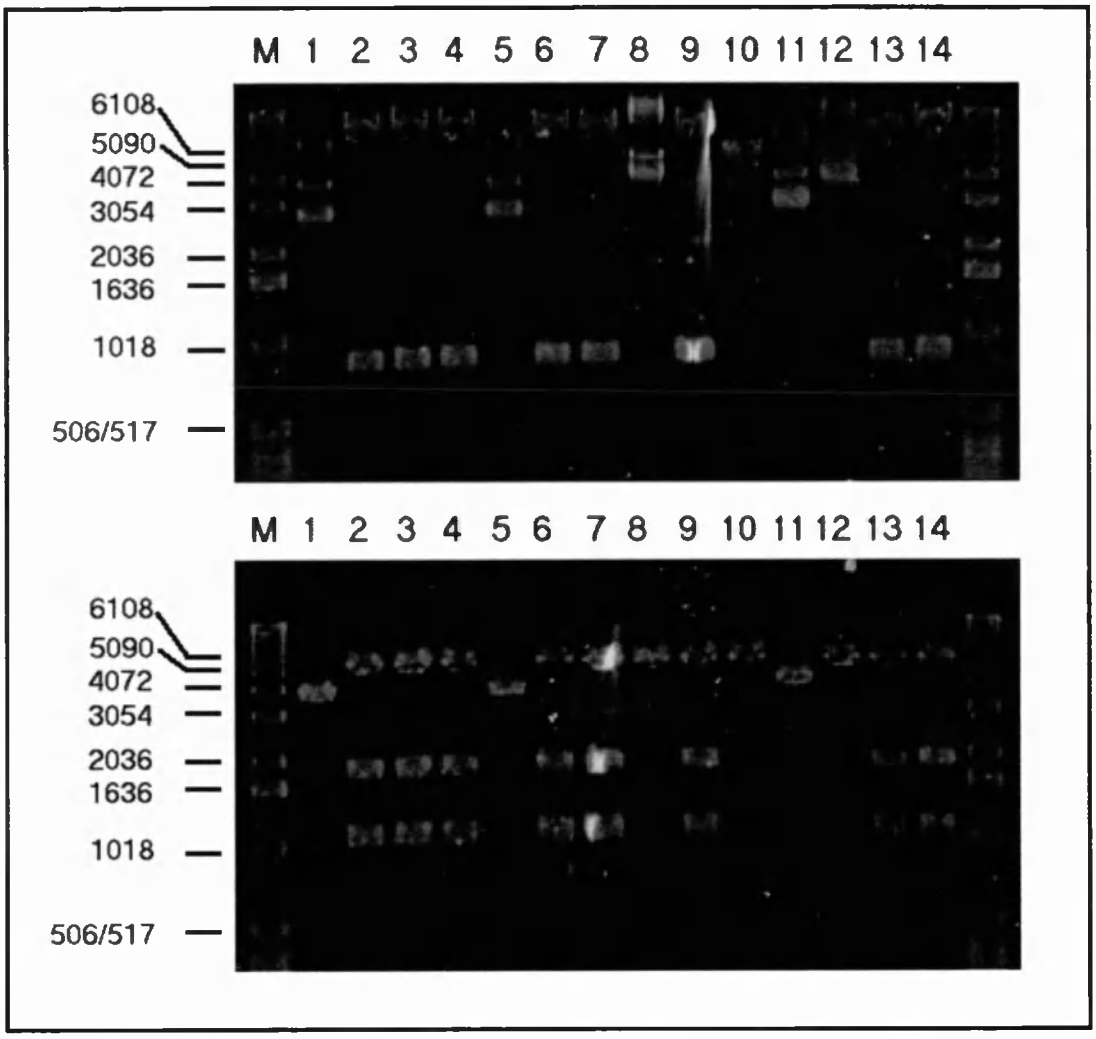


Fig. 8.8.2. Restriction enzyme analysis of positive clones MT-1 in pcDNA3.1$^{(+)}$myc/His. Positive clones were analysed by restriction digest with either Xho I (upper lanes1-14) or Hind III (lower lanes1-14). The expected digestion products of Xho I were 7.5kbp and 820bp and for Hind III; 5.3kbp, 1.9kbp and 1.1kbp. Relevant marker sizes are indicated and DNA was visualised by staining with 1.0μg ml^{-1} ethidium bromide.

Eight positive clones were identified (Fig. 8.8.2., lanes 2, 3, 4, 6, 7, 9, 13 and 14) and one clone was selected for sequence analysis. Sequence analysis of the selected clone required detailed analysis of the C-terminus to confirm that c-*fms* had been cloned in frame with the myc/His epitopes. Therefore the entire c-*fms* coding region was sequenced in both forward and reverse orientations to confirm that the sequence would code for full length c-*fms*. This was carried out using gene overlapping, gene specific primers (Sequencing primers, appendix C) to generate a consensus sequence for MT-1 (Appendix E). The consensus sequence was compared with the published

protein sequence of human c-*fms* (Swiss-Prot. Accession No. P07333) using a tBlastx sequence similarity program (Appendix F). The consensus sequence for MT-1 translated into a 1014 amino acid protein containing the 972 amino acids of c-*fms* and read-through the mutagenised stop codon into the *myc*/His epitope present immediately upstream of a stop codon (Appendix F). Therefore MT-1 contained the full length c-*fms* coding region with a C-terminal epitope tag in a mammalian expression vector under the control of the CMV promoter. This construct now provided the basis for site directed mutagenesis studies to introduce mutations within the c-*fms* coding sequence.

8.9.1. Introduction of single amino acid substitutions into MT-1 by site directed mutagenesis

The epitope tagged human c-*fms* construct produced in chapter 8 was used as a template for the introduction of single amino acid substitutions at three residues. Mutations were introduced at two tyrosine residues, Tyr^{708} and Tyr^{723}, in the kinase insert region of c-*fms* by altering the nucleotide composition at the relevant tyrosine residue to code for a phenylalanine by site directed mutagenesis using the Stratagene Quick-Change SDM protocol (Chapter 3.4.14.). Mutagenesis was accomplished from supercoiled, dsDNA, MT-1 using two synthetic oligonucleotide primers containing the desired mutation. The oligonucleotide primers, each complementary to opposite strands of the vector, were extended by PCR to synthesise a mutated plasmid containing staggered nicks. After amplification, the PCR product was digested with the methylated DNA sensitive restriction enzyme, Dpn I which preferentially digested the methylated, parental DNA template, selecting for the synthesised DNA containing the desired mutation. The nicked vector DNA incorporating the desired mutations was

then transformed into *E. coli.* Table 8.9.1. summarises the various c-*fms* mutant constructs synthesised.

Site directed mutagenesis of Tyr^{708} was carried out with primers Y708F-1 and Y708F-2 and mutagenesis of Tyr^{723} was carried out with primers Y723F-1 and Y723F-2 to generate MT-2 and MT-3, respectively (Appendix C). Following transformation of *E. coli*, clones were screened by sequence analysis with the diagnostic primer set SF-3 and SR-6, and clones containing each point mutation were obtained (Appendix G). The double mutation construct, MT-4, was produced by repeating the mutagenesis on an MT-2 clone with primers Y723F-1 and Y723F-2. Again, following transformation of *E. coli*, clones were screened by sequence analysis with the diagnostic primer set SF-3 and SR-6, and clones containing both point mutations were identified (Appendix G).

In addition to mutation of the tyrosine residues involved in PI 3-kinase activation a single leucine residue at Leu^{301}, in the fourth Ig-like loop of c-*fms* was mutated to serine. Mutation of this site had previously been demonstrated to confer transforming capability on c-*fms* expressed in NIH 3T3 cells (Roussel et al., 1988). Site directed mutagenesis was carried out using two homologous primers, L301S-1 and L301S-2 (Appendix C). Three constructs were generated from clones MT-1, MT-2 and MT-3 containing an amino acid substitution at Leu^{301} to give MT-5, MT-6 and MT-7, respectively (Table 8.9.1.). The clone MT-5 contained a single mutation at Leu301 and coded for a transformed, wild-type epitope tagged human c-*fms*. Clones MT-6 and MT-7 contained the Leu301Ser mutation in addition to mutations phenylalanine mutations at Tyr^{708} and Tyr^{723}, respectively. Following transformation of *E. coli*, each

clone was sequenced with the diagnostic primer set SF-1 and SR-3, and clones containing the single point mutation were identified (Appendix G).

	Leu^{301}Ser	**Tyr^{708}Phe**	**Tyr^{723}Phe**
MT-1	-	-	-
MT-2	-	+	-
MT-3	-	-	+
MT-4	-	+	+
MT-5	+	+	-
MT-6	+	-	+
MT-7	+	-	-

Table 8.9.1. Summary of human c-*fms* mutant constructs.

8.10.0. Discussion

The aim of this chapter was the construction of a c-*fms* construct for introduction into mammalian cells for expression of a full length tagged c-*fms*. A variety of cloning approaches were employed in an attempt to clone c-*fms* successfully into pCDNA3.1 based vectors. This initial strategy avoided the amplification of the entire coding region, which may have introduced undesired errors and also exploited the presence of convenient restriction sites (Fig. 8.1.0.).

The initial strategy involved PCR amplification of the 3' portion of c-*fms* from a cDNA template, pZipSV(X)/c-*fms*, either as a homologous product (3'PCR1) or as a product with the c-*fms* stop codon replaced with a Hind III site (3'PCR2). The 5' end of these PCR products contained an internal Xho I site that facilitated the ligation of the PCR product with the remainder of the 5' end of c-*fms* (5'RF), liberated from the cDNA template by digestion with Eco RI and Xho I. Both the 5'RF and 3'PCR1 were to be cloned, in tandem, into pcDNA3.1$^{(-)}$, to produce WT-1 and the 5'RF and 3'PCR2 were to be cloned, in tandem, into pcDNA3.1$^{(-)}$*myc*/His, to produce MT-1. After extensive optimisation of PCR conditions the 820bp 3'PCR products were successfully amplified from cDNA template and their sequence was confirmed. The 5'RF was also liberated successfully from pZipSV(X)/c-*fms*. Ligation of both 5'RF and 3'PCR2 into pcDNA3.1$^{(-)}$*myc*/His was attempted, however although cloning conditions had been optimised, cloning did not succeed. Therefore it was not possible to continue cloning c-*fms* into the available pcDNA3.1 vectors by this approach, and cloning each fragment into pcDNA3.1 separately was also not possible due to the negative orientation of the MCS which did not contain restriction enzyme sites in a suitable location.

A modified strategy was employed to circumvent the limitations of the pcDNA3.1 MCS. This involved using a shuttle vector for cloning together of each fragment before subcloning the entire c-*fms* gene into the relevent pcDNA3.1 vector (Fig. 8.6.1.). This strategy was essentially the same as the original methodology, and utilised the already cloned 3'PCR2 and the 5'RF products. However, this approach allowed cloning of the 5'RF into a pGEM based cloning vector which had an Eco RI site upstream of Xho I in the MCS. In addition once the 5'RF was cloned into the pGEM vector, the 3'PCR2 product could then be inserted downstream of the 5'RF on Xho I and Hind III. The final product of these ligation reactions would then have been liberated from pGEM after Eco RI Hind III digestion then cloned directly into pcDNA3.1$^{(-)}$*myc*/His (Fig. 8.6.1.). A similar approach would also have been employed for construction of WT-1.

Although the 3'PCR2 fragment was successfully cloned into pGEM11Zf(+), repeated attempts at cloning 5'RF into pGEM3'PCR on Eco RI and Xho I failed. A diagnostic restriction digest revealed the presence of a Hind III site in the 5'RF. A new search of the published sequence did reveal a Hind III site at 1349bp in the human c-*fms* mRNA sequence. Why this site had not been detected initially is unclear, however the published c-*fms* sequence has been updated and it is possible that there had been an error in the sequence originally analysed for Hind III. The presence of this Hind III site explained why identification of clones containing the c-*fms* gene was confusing. In addition, the Hind III site at 1349bp also prohibited subcloning of WT-1 and MT-1 into pcDNA3.1 vectors as had been intended.

At this point an alternative cloning strategy had to be devised that did not rely on unique restriction enzyme sites. The recent availability of pcDNA3.1 based vectors

with a MCS in the positive orientation facilitated the subcloning of a full-length c-*fms* PCR product. Amplification of c-*fms* could now be accomplished with proof-reading DNA polymerases that had greatly reduced error rates compared to *Taq* DNA polymerase, used for the original cloning attempts. Therefore the full length c-*fms* coding sequence was amplified from pZipSV(X)/c-*fms* either as a completely homologous product (WT-1), or with the stop codon re-engineered to a Hind III site (MT-1). The two full-length c-*fms* amplification products were cloned into pCR2.1 and positive clones identified by restriction digest analysis. WT-1 positive clones were digested with Eco RI and WT-1 was subcloned into pcDNA3.1$^{(-)}$. The 5' and 3' ends of two positive clones obtained from this ligation were sequenced to confirm the orientation of WT-1 in pcDNA3.1$^{(-)}$ (Appendix D).

MT-1, liberated from pCR2.1 was successfully subcloned into pcDNA3.1$^{(+)}$ *myc*/His on Bam HI and Not I. Sequence analysis of the 5' and 3' ends of two positive clones obtained from this ligation confirmed that they contained MT-1 (Data not shown) and more detailed sequence analysis of one clone, using contiguous gene specific primers in both orientations, confirmed that the expression vector contained full length c-*fms*, that read through into the *myc*/his tag (Appendices E).

The WT-1 and MT-1 constructs represented two novel expression constructs for exogenous expression studies of c-*fms* in mammalian cells. Both the WT-1 and MT-1 constructs were designed for high levels of protein expression in mammalian cells under the control of the cytomegalovirus promoter (CMV). In addition, MT-1 had the additional advantage of a *myc*/His tag sequence at the C-terminus. Fig. 8.10.1. is a schematic diagram of the cloned c-*fms* expression constructs and positions of the mutations on MT-1 introduced through site directed mutagenesis.

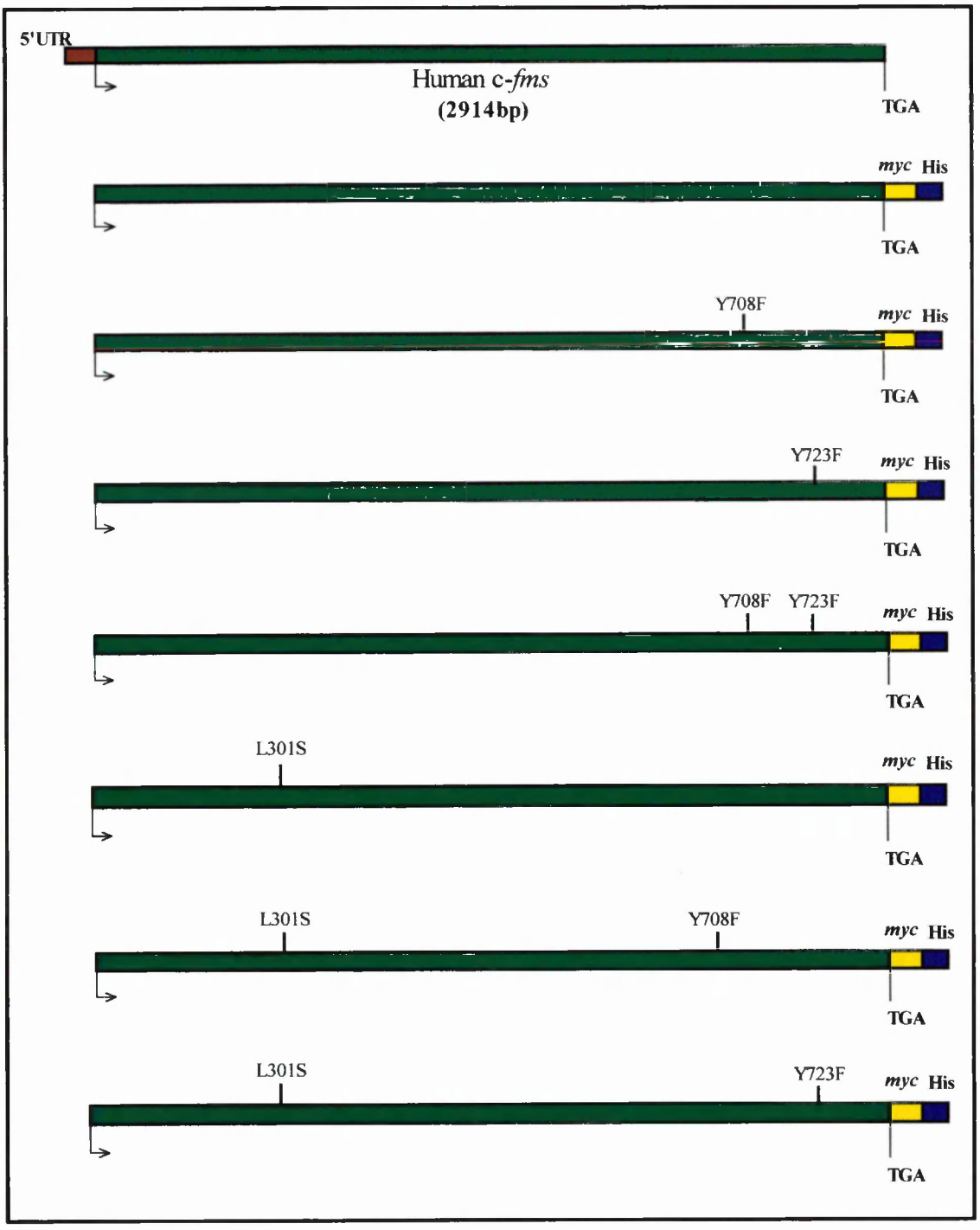


Fig. 8.10.1. Diagrammatic representation of the WT-1 and MT-1 c-*fms* constructs in mammalian expression vectors.

The *myc* epitope will facilitate detection of expressed protein with specific antibodies that recognise the *myc* 9E10 epitope. An alternative to c-*fms* specific antibody detection will also be preferable for discriminating between endogenous c-*fms* and recombinant expression and this is also facilitated by the *myc* epitope. The His tag

will also facilitate detection of recombinant protein as well as high-affinity purification.

The MT-1 construct was also used as a template for site directed mutagenesis of key residues in the c-*fms* extracellular and cytoplasmic domains. A series of c-*fms* constructs, mutated at a combination of Leu^{301}, Tyr^{708} and Tyr^{723} were produced (Table 9.5.1. and Fig. 8.11.1.). The MT-2 mutant contains a Tyr708Phe mutation, MT-3 contains a Tyr723Phe mutation and MT-4 contains mutations at both sites. Each construct was engineered for the investigation of PI 3-kinase binding to c-*fms*. In addition, mutation of Leu301Ser in the extracellular domain was carried out on MT-1, MT2 and MT-3, to produce MT-5, MT-6 and MT-7, respectively. Constructs containing the Leu301Ser mutation on c-*fms* possess transforming capability and are expected to be constitutively activated in transfected cells.

CHAPTER 9

Characterisation of human c-*fms* epitope tagged constructs expressed in mammalian cells.

9.0.0. Introduction

Exogenous expression of c-*fms* in a variety of mammalian cell lines has generated a substantial amount of conflicting data. This includes conflicting data regarding STAT activation, where the activation of Jak kinases in c-*fms* transfected fibroblasts was not observed in BAC1.2F5 macrophages (Novak et al., 1995). In addition, p120ras•GAP is phosphorylated and associates with p190Rho•GAP in v-*fms* transformed fibroblasts (Trouliaris et al., 1995), however p120ras•GAP phosphorylation was not observed in BAC1.2F5 macrophages (Reedijk et al., 1990).

It has also been difficult to ascertain whether M-CSF stimulated responses in macrophages require PI 3-kinase. C-*fms* transfection studies, when considered together, only suggest that different cell lines provide a different environment of signalling molecules for c-*fms* signalling. For example, data obtained early on in c-*fms* transfection studies suggested that mutation of Tyr^{721} in the murine receptor when expressed in Rat-2 fibroblasts, resulted in a concomitant loss of PI 3-kinase activity and cell proliferation (Reedijk et al., 1992) which was also suggested by a study carried out in NIH 3T3 fibroblasts with a deleted c-*fms* KI domain (van der Geer and Hunter, 1993). However, in FDC-P1 myeloid cells transfected with a c-*fms* mutant lacking the KI domain, M-CSF continued to stimulate cell proliferation comparable to wild type c-*fms* (Kanagasundaram et al., 1996). These examples emphasise the need for a more relevant macrophage cell or transfection model in which c-*fms* signalling can be disseminated without the drawbacks observed with classical cell lines routinely chosen for transfection studies.

It is evident that common transfectable cell lines possess unique properties which are not consistent with those found in macrophages. Although the various components of

a signalling pathway may be present this alone does not guarantee that c-*fms* signalling can be replicated. Other requirements might include the presence of correct signalling complexes, the correct subcellular localisation of signalling molecules as well as the correct numbers of molecules per cell.

Therefore to examine c-*fms* signalling within the context of macrophages, a macrophage cell line must be utilised. It would be an advantage to express c-*fms* DNA constructs in a relevant cell model such as BAC1.2F5 cell line. Studies of mutant c-*fms* constructs, over-expressed in BAC1.2F5 cells in a dominant negative manner, would facilitate further analysis of M-CSF stimulated responses in BAC1.2F5 cells which have now been extensively characterised, that rely on the direct association of PI 3-kinase with phosphorylated c-*fms*.

However, it has been demonstrated that established cell lines of the haematopoietic lineage such as T-cells and macrophages are notoriously difficult cells in which to express exogenous DNA. It has been demonstrated in this laboratory that T-cells are readily transfectable with commonly available reagents, but detection of protein expression has either been very poor or undetectable. In some instances activation of T-cells with phorbol esters has upregulated protein expression although this method of cell activation is undesirable for studying specific signalling pathways. Similar findings have also been reported for macrophage cell lines and the available published data on macrophage transfection suggests that viral based transfection systems are the most commonly used methods for attaining high levels of exogenous protein expression. Alternative means for introduction of protein into macrophages includes micro-injection which has proved successful for a number of groups (Allen et

al., 1997). However micro-injection is a laborious technique and for biochemical studies of receptor mutants is an impractical approach.

Therefore the overall aim of this chapter was to transfect the c-*fms* constructs produced in chapter 8, into BAC1.2F5 macrophages. This involved determination of a viable transfection system for macrophages and a number of commercial systems were investigated. Following identification of a suitable transfection methodology, the c-*fms* constructs would be introduced into BAC1.2F5 cells and once c-*fms* expression had been accomplished, a detailed biochemical and functional study was intended.

The presence of epitope tags at the C-terminus of each mutant construct would not only facilitate identification of exogenous receptor from endogenous receptor, but also the selective precipitation of human c-*fms* and the co-precipitation of signalling molecules associated with exogenous receptor molecules. Differences in the association of PI 3-kinase with mutated c-*fms*, could then be correlated to the observed differences in M-CSF stimulated responses in transfected BAC1.2F5 cells compared to untransfected cells.

9.1.0. Characterisation of efficient transfection protocols for the introduction of DNA into BAC1.2F5 macrophages

Many methods exist for the introduction of DNA into mammalian cells. These include calcium phosphate, electroporation and lipid mediators (Graham and Eb AJ van, 1973; Neumann et al., 1982; Rizzo et al., 1983). To date, there are few published protocols available which describe the successful transfection and expression of exogenous proteins in macrophage cells. In order to express the c-*fms* constructs in BAC1.2F5 cells the most favourable transfection method was determined by comparing various transfection techniques.

Several protocols were analysed for efficiency of transfection using a wild type enhanced Green Fluorescent Protein (WT eGFP) construct (appendix C) into BAC1.2F5 cells. Efficiency of transfection was measured as a percentage of the cell population expressing eGFP, measured by FACS. Four lipid based methods, LipoFectAMINE™ (Gibco-BRL), LipoFectin™ (Gibco-BRL), CellFectin™ (Gibco-BRL) and DIMRIE-C™ (Gibco-BRL), a Calcium Phosphate based method (ProFection™, Promega) and a poly-cationic method, SuperFect™ (Qiagen) were compared for their transfection efficiency (Chapter 3.4.17.).

LipoFectin™, CellFectin™ and DIMRIE-C™ lipid-mediated transfection techniques all led to high cell mortality which resulted in a harvested cell population that was too small for analysis by FACS (Data not shown). LipoFectAMINE™, ProFection™ and SuperFect™ transfection methods were less toxic to cells and therefore FACS analysis was possible (Figs. 9.1.1.).

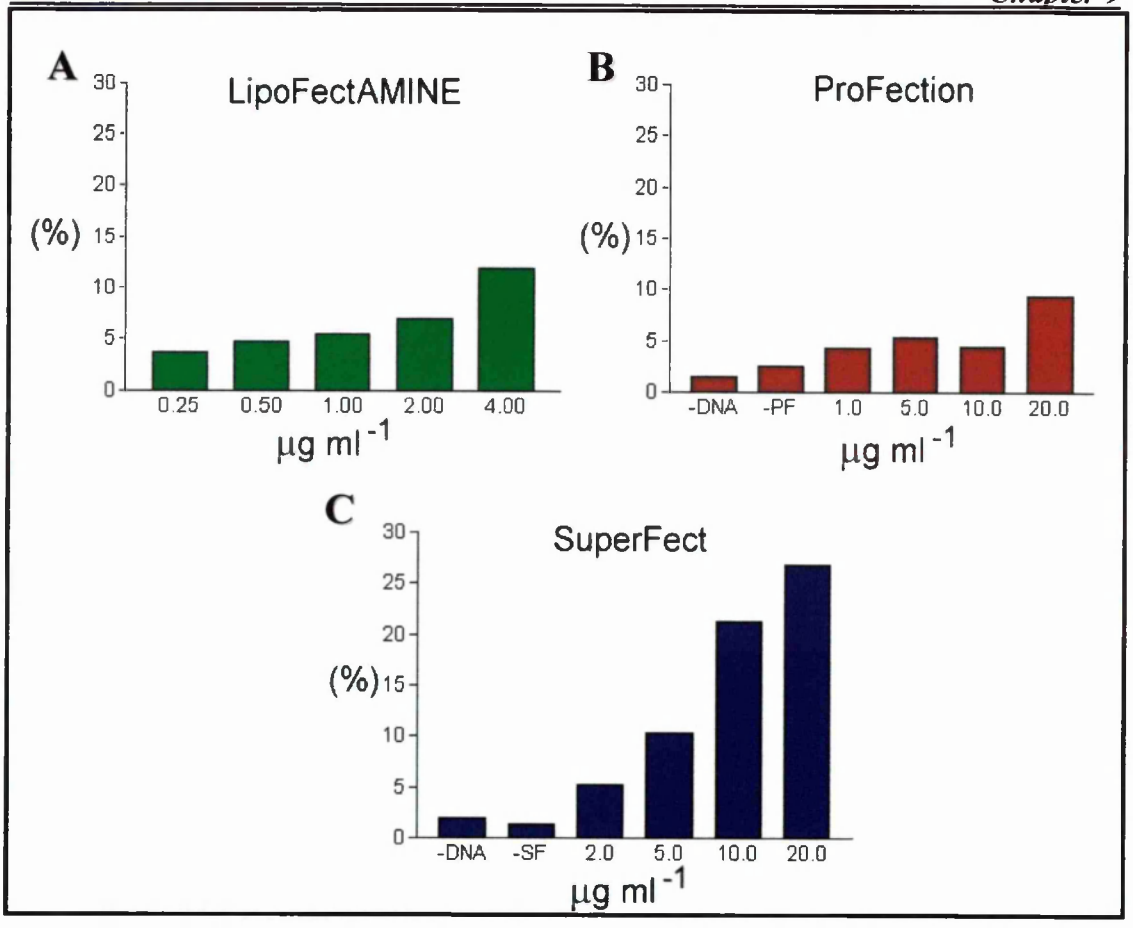


Fig. 9.1.1. Transfection efficiency of wild-type eGFP construct into BAC1.2F5 cells. Three alternative transfection methods were compared directly for efficiency of transfection of a WT eGFP construct (Clontech). Cells were harvested 24 hours post-transfection and analysed by FACS. Panel A is the lipid-mediated LipoFectAMINE™ (Gibco-BRL), panel B is the Calcium Phosphate-based ProFection™ and panel C is the polycationic-based SuperFect™ (Qiagen).

The highest transfection efficiency was observed with SuperFect™ at 26%, however this required the greatest concentration of DNA, (20µg ml^{-1}). However, at comparable DNA concentrations ProFection™ had only 10% transfection efficiency, which suggested that SuperFect™ would be the more suitable choice for transfection of BAC1.2F5 cells if DNA was readily available. Interestingly, lipid-mediated transfection protocols were shown to be highly toxic to BAC1.2F5 cells, however LipoFectAMINE™ appeared to be the least toxic and was capable of achieving a reasonable transfection efficiency (13%) with 4 µg ml^{-1} DNA, which was more efficient than SuperFect with 5 µg ml^{-1} DNA. Therefore, because of the low toxicity

and high transfection efficiency, SuperFect™ was chosen as the method of choice for transfection studies in BAC1.2F5 cells where recombinant DNA was readily available. In the event that only small quantities of DNA were available for transfection purposes, LipoFectAMINE™ was also considered.

9.2.0 Expression of WT- and MT-1 in BAC1.2F5 macrophages

The SuperFect™ transfection conditions, optimised in the previous section, were used to introduce both WT-1 and MT-1 into BAC1.2F5 cells. Endotoxin free plasmid DNA (Chapter 3.4.10) was transfected into BAC1.2F5 cells plated out at $1.5x10^{-4}$ per 13mm round coverslip or at $1.0x10^{-5}$/well on 6-well tissue culture dishes. Transient transfections were examined either biochemically by western blot or immuno-fluorescent analysis with gene specific and *myc*-epitope specific antibodies. Repeated attempts to detect expression of either WT-1 or MT-1 in BAC1.2F5 cells transfected by either optimised SuperFect or LipoFectAMINE transfection methods failed (Data not shown). Detection of expressed protein may have been hindered by either a low transfection efficiency or poor protein expression which was below detection sensitivity. It must be noted that the transfection conditions had been optimised with an eGFP-based construct, expression of which in mammalian cells is regulated by the same CMV promoter found in pcDNA3.1 based vectors. However, detection of eGFP expression was carried out by FACS analysis, a more sensitive detection method than that used for detection of WT-1 and MT-1. In other cell lines, e.g. T-cells, expression from the CMV promoter requires cell activation with Phorbol 12-Myristate 13-Acetate (PMA). Transiently transfected BAC1.2F5 cells, 18 hours post transfection were incubated for 5 hours with 10nM PMA prior to analysis of MT-1 expression by western blot analysis with gene specific antibodies. PMA activation of BAC1.2F5

cells did not upregulate expression of exogenous c-*fms* to a detectable level (Data not shown).

9.3.0. In vitro translation of MT-1

In order to confirm that the c-*fms* coding sequence in the MT-1 expression construct had the capacity to code for full length c-*fms*, MT-1 was translated, *in vitro*, using both a Rabbit Reticulocyte and Wheatgerm Agglutinin *in vitro* translation system (Chapter 3.4.16.). The ^{35}S methionine labelled translation products from MT-1 were resolved by SDS-PAGE and detected by autoradiography (Fig. 9.3.1.). A single product of approximately 130-140kDa was detected in both *in vitro* translation systems (Fig. 9.3.1.).

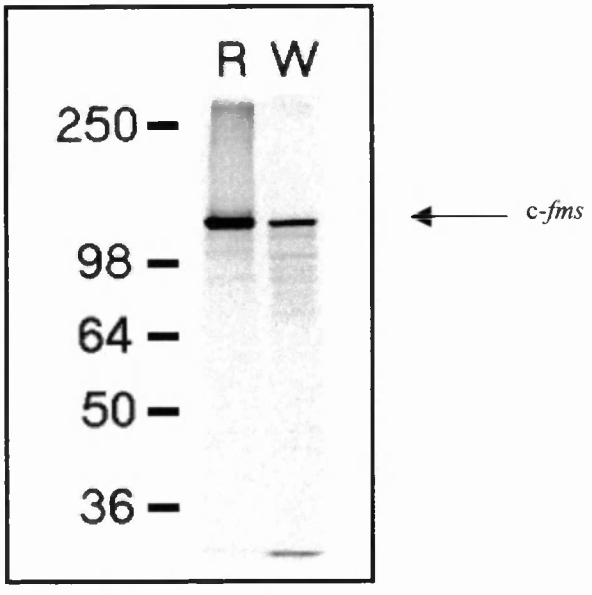


Fig. 9.3.1. SDS-PAGE analysis of an *in vitro* translation of human, epitope tagged c-*fms* (MT-1). Lane R is a rabbit reticulocyte based *in vitro* translation reaction and lane W is a comparative wheatgerm agglutinin *in vitro* translation. Membranes were exposed to autoradiography film for 22 hours.

The M_r of the immature, human c-*fms* is approximately 130kDa, the additional 42 amino acids corresponding to the C-terminal linker and epitope tags, when taken into account, would increase the M_r to approximately 135kDa which is comparable to that observed in the *in vitro* translation. The data from the *in vitro* translation indicates

that MT-1 is capable of translating a full length product containing the entire human c-*fms* coding region and the C-terminal *myc*/His epitope tags.

9.4.0. Transient expression of MT-1 in HEK 293 epithelial cells detected by western blot analysis

In order to validate MT-1 further, it was necessary to express this construct in a readily transfectable mammalian cell line. For this purpose the human embryonic kidney epithelial cell line, HEK 293 was chosen. This cell line has previously been demonstrated to be readily transfectable by Calcium Phosphate and have been shown to express exogenous protein at relatively high levels (Yang et al., 1998). Therefore to validate MT-1, HEK 293 cells were transfected using the ProFection™ Calcium Phosphate transfection protocol (Chapter 3.4.17.). Cells were lysed at 24 and 72 hours post-transfection and analysed by SDS-PAGE. Western blot analysis demonstrated that tagged c-*fms* (MT-1) was readily detected with anti-*myc* epitope antibody in whole cell lysates from transiently transfected HEK 293 cells (Fig. 9.4.1.).

Tagged c-*fms* expression was detected as early as 24 hours post transfection (Fig. 9.4.1., lanes 3 and 4). At 24 hours post transfection tagged c-*fms* was expressed as two isoforms of 135kDa and 155kDa, corresponding to the immature and mature receptor forms, respectively (Fig. 9.4.1., lanes 3 and 4). These receptor bands are comparable to those previously been observed for the murine receptor (Chapter 4.2.1.) and reported for the human receptor (Sherr et al., 1990). However, the immature and mature tagged c-*fms* isoforms exhibit an increased M_r on SDS-PAGE due to the presence of the *myc*/His tags (Fig. 9.4.1., lanes 3 and 4). The immature form of tagged c-*fms* is the predominant isoform present at 24 hours post transfection, and 72 hours it appears to be the only isoform present (Fig. 9.4.1., lanes 5 and 6). The 65

kDa band, present in both untransfected and transfected cell lysate (Fig. 9.4.1.) corresponds to endogenous c-*myc* (Persson and Leder, 1984).

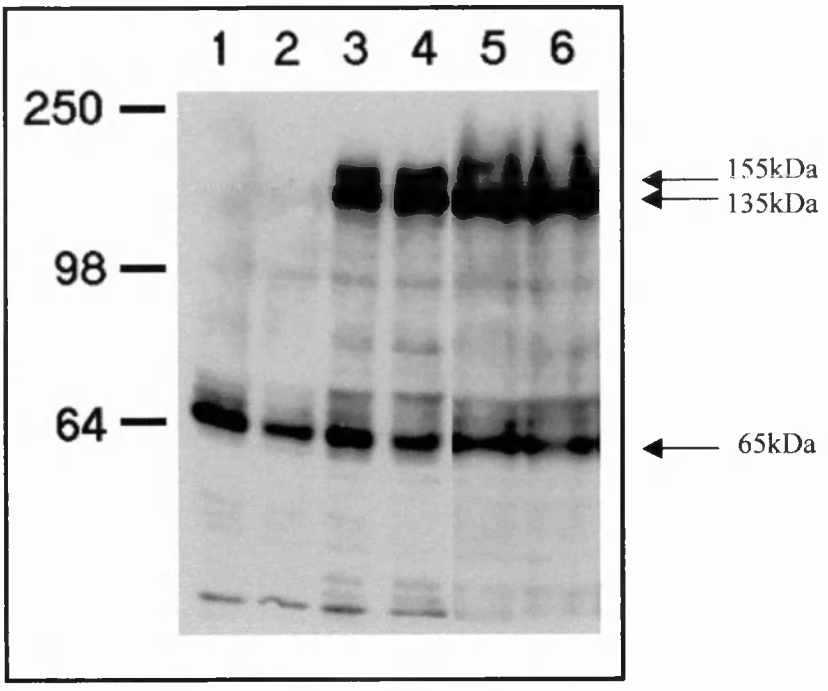


Fig. 9.4.1. SDS-PAGE analysis of whole cell lysate from HEK 293 cells transiently transfected with MT-1 . Approximately 10μg of total cell lysate proteins were resolved by 7.5% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-*myc* epitope mAb (Calbiochem). Lanes 1 and 2 represent lysates from no DNA and no Calcium phosphate reagent controls, respectively. Lanes 3 and 4 are lysates from duplicate transient 24 hour transfections (4μg ml^{-1}) and lanes 5 and 6 are comparable transfections (4μg ml^{-1}) at 72 hours.

Expression of tagged c-*fms* was also characterised by immunoprecipitation with an anti-*myc* epitope antibody to demonstrate that the epitope tag would facilitate purification of c-*fms* from transfected cells. HEK cells, transiently transfected with tagged c-*fms*, were harvested at 24 hours post transfection and tagged c-*fms* was immunoprecipitated with an anti-*myc* epitope antibody directly conjugated to agarose (Santa Cruz). SDS-PAGE analysis of immunoprecipitated tagged c-*fms* demonstrated that both immature and mature tagged c-*fms* were purified from HEK cell lysate and

could be detected with an anti-*myc* antibody (Santa Cruz) (Fig. 9.4.2., Panel A, lanes 3-5).

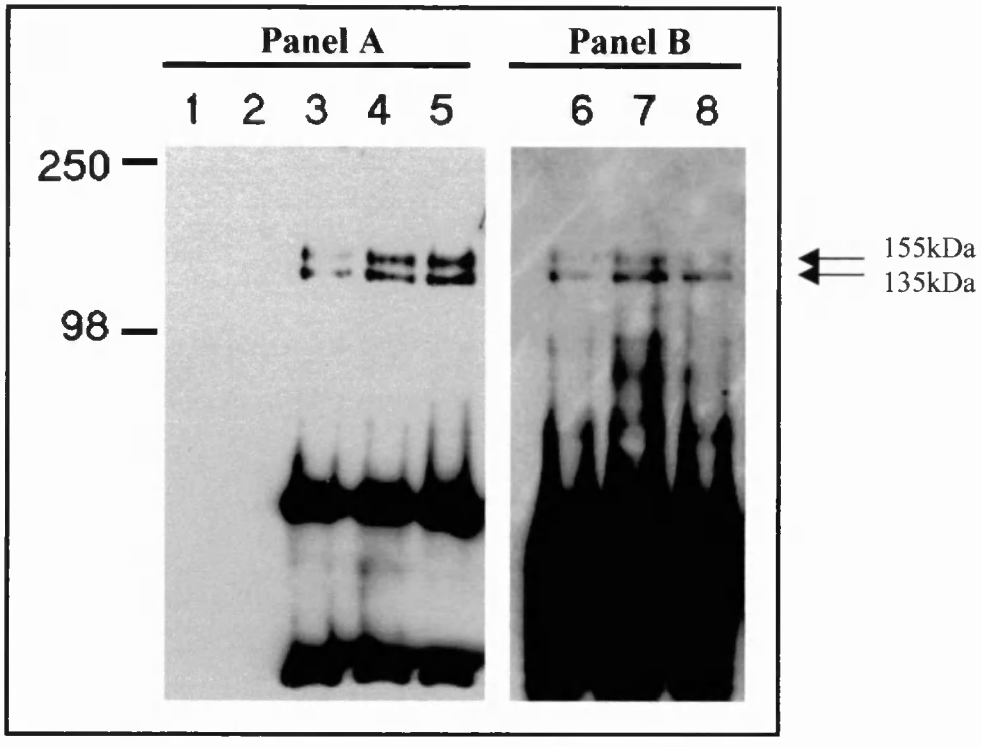


Fig. 9.4.2. SDS-PAGE analysis of protein immunoprecipitated HEK 293 cells transiently transfected with MT-1. Tagged c-*fms* was immunoprecipitated with an agarose-conjugated anti-*myc* mAb (Santa Cruz). Precipitated proteins were resolved by 7.5% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with either an anti-*myc* epitope mAb (Calbiochem) (Panel A) or an anti-human c-*fms* pAb (Santa Cruz) (Panel B). Lanes 1 and 2 represent lysates from no DNA and no Calcium phosphate reagent controls, respectively. Lanes 3 → 8 are lysates from transient 24 hour transfections (4μg ml^{-1}).

In addition, an antibody specific for the extracellular domain of human c-*fms* (Santa Cruz) was also capable of detecting immunoprecipitated tagged c-*fms* (Fig. 9.4.2., Panel B, lanes 6-8).

9.5.0. Immunofluorescent analysis of HEK cells transiently expressing MT-1

To determine whether tagged c-*fms* expression could be detected by immunofluorescence, transiently transfected HEK cells were probed with c-*fms*-specific and *myc* epitope-specific antibodies (Fig. 9.5.1.). Tagged c-*fms* transiently transfected into HEK cells was detected with both c-*fms* (Fig. 9.5.1., top panels) and c-*myc* (Fig. 9.5.1., bottom panels) epitope specific antibodies at 24 hours.

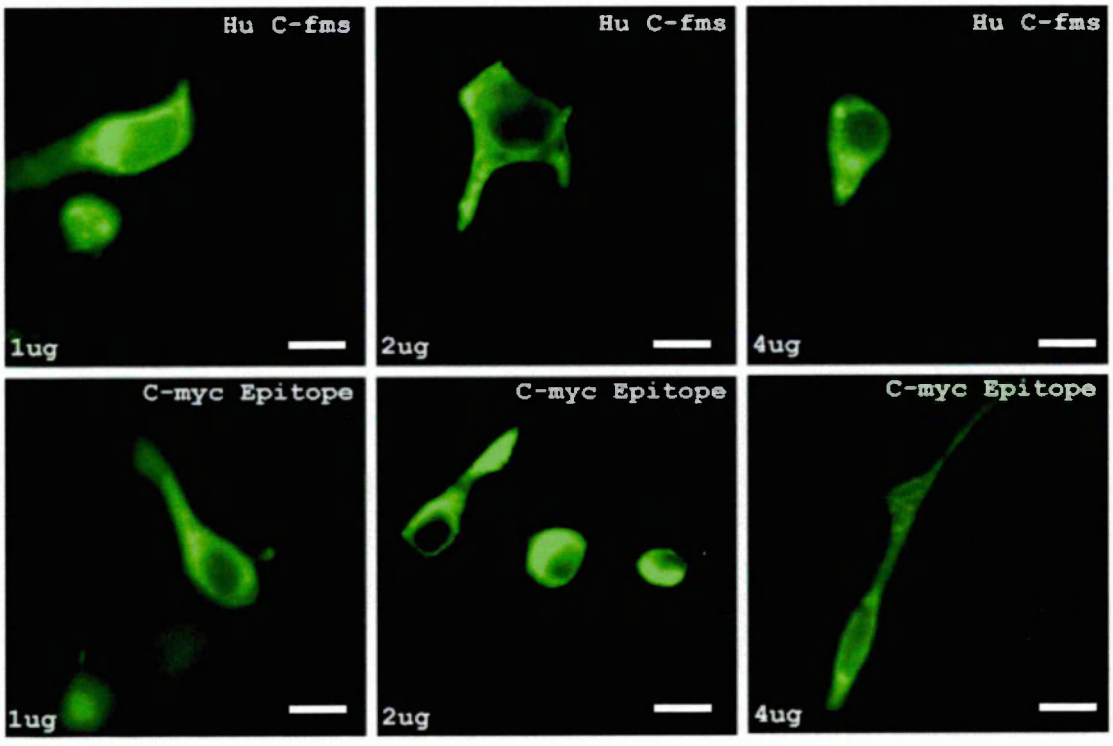


Fig. 9.5.1. Immunofluorescent analysis of MT-1 expressed in HEK cells. Transiently transfected HEK cells were probed either with an anti-human c-*fms* (top panels) or anti-c-*myc* epitope (bottom panels) antibody and visualised with an Alexa^{488}-conjugated secondary antibody (Molecular Probes). Each panel is representative of at least three separate experiments. Bars indicate 10μm.

These data confirm that c-*fms* localisation can be detected by immunofluorescence and that both gene specific as well as epitope specific antibodies can readily detect exogenous protein. Further analysis of tagged c-*fms* transfected HEK cells for the subcellular localisation of tagged c-*fms* suggests that c-*fms* is localised to the plasma membrane and to discrete cytosolic locations 24 hours post transfection (Fig. 9.5.2.).

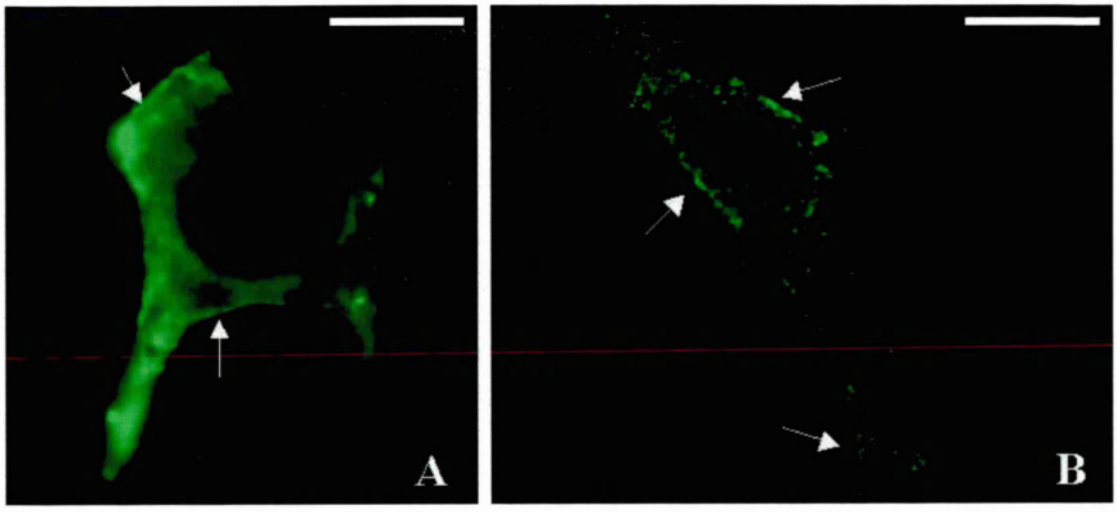


Fig. 9.5.2. Immunofluorescent analysis of MT-1 expression in HEK cells. Transiently transfected HEK cells were probed with an anti-human c-*fms* antibody and stained with an Alexa^{488}-secondary antibody. Panel A is single positive HEK cell recorded by photomicrography. Panel B is a single 1μm deconvolved section of a comparable cell captured with a digital camera and processed by OpenLab 1.7.8. Bars indicate 10μm.

At higher magnification, the plasma membrane localisation of tagged c-*fms* can be visualised with an anti c-*fms* antibody as illustrated by panel A of Fig. 9.5.2. in 24 hour transient transfections. The white arrows in panel A clearly indicate plasma membrane staining whilst the nucleus is clearly delineated and the cytosol contains very little positive staining (Fig. 9.5.2.). This is the anticipated staining pattern of c-*fms* localisation and confirms that tagged c-*fms* is localised correctly in transfected HEK cells. Using a digital analysis package (OpenLab 1.7.8, Improvision) which allows software enhancement of a fluorescent specimen, a single 1μm section through a positively stained, tagged c-*fms* transfected HEK cell (Fig. 9.5.2., panel B) revealed predominant plasma membrane staining (white arrows) as well as punctate cytosolic staining which extended throughout the cell. Therefore immunofluorescent detection of the expressed tagged c-*fms* construct in HEK cells confirmed that c-*fms* is localised at the plasma membrane as expected for a cell surface receptor.

Since the expression pattern of tagged c-*fms* isoforms appears to vary from 24 hours to 72 hours post transfection as detected by western blot analysis, it seemed likely that this would also affect the subcellular localisation of tagged c-*fms*. Analysis of cells 72 hours post-transfection indicated that tagged c-*fms* is not localised at the plasma membrane but instead is found predominantly in the cytosol (Fig. 9.5.3.). Investigation of tagged c-*fms* localisation with digital image analysis revealed that tagged c-*fms* was localised in distinct pockets around the nucleus (Fig. 9.5.3., panels A and B). In panel A of Fig. 9.5.3. the white arrow indicates the positively stained cytosolic location of tagged c-*fms* in a single 1μm transverse section through a transfected HEK cell. For comparison the location of the plasma membrane of a transfected HEK cell is indicated in panel B of Fig. 9.5.3.. It is evident that at 72 hours post transfection tagged c-*fms* is no longer expressed at the plasma membrane.

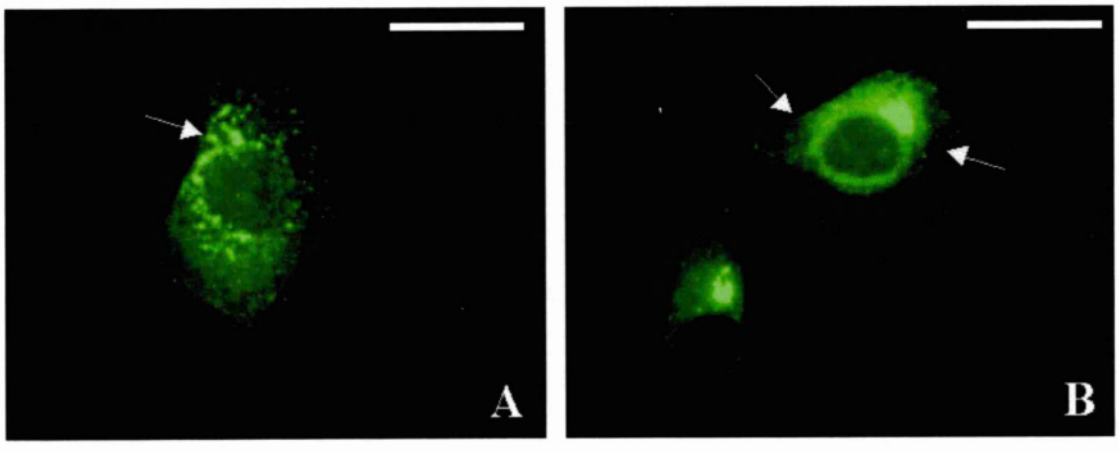


Fig. 9.5.3. Immunofluorescent analysis of MT-1 expression in HEK cells after 72 hours. Transiently transfected HEK cells were probed with an anti-*myc* epitope antibody and stained with an Alexa^{488}-secondary antibody. Panels A and B are single 1μm deconvolved sections of a comparable cells captured with a digital camera and processed by OpenLab 1.7.8. Bars indicate 10μm.

9.6.1. Transient expression of mutant epitope tagged c-*fms* in HEK cells

To determine whether mutation at either Tyr^{708} or Tyr^{723} in the cytoplasmic tail affected tagged c-*fms* expression or subcellular localisation, two expression

constructs, MT-2 and MT-3, were transiently transfected into HEK cells. Transient expression of MT-2 which harbours a Y708F mutation, and MT-3 which harbours a Y723F mutation in HEK cells was detected by western blot analysis (Fig. 9.6.1.).

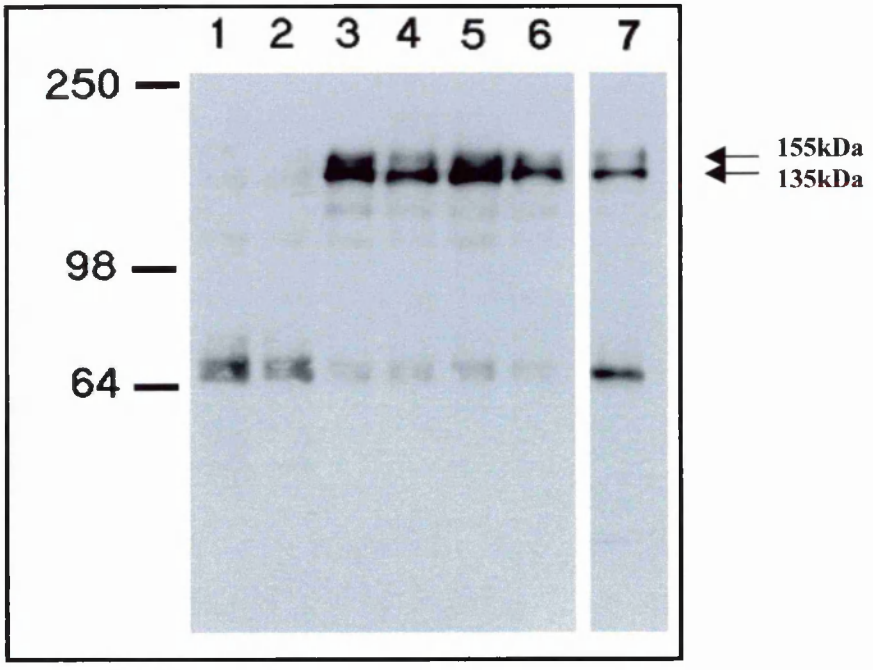


Fig. 9.6.1. SDS-PAGE analysis of whole cell lysate from HEK 293 cells transiently transfected with MT-2 and MT-3. Approximately 10μg of total cell lysate proteins harvested at 48 hours post-transfection were resolved by 7.5% SDS-PAGE and then blotted onto a nitrocellulose membrane prior to immunostaining with an anti-*myc* epitope mAb (Calbiochem). Lanes 1 and 2 represent lysates from no DNA and no calcium phosphate reagent controls, respectively. Lanes 3 and 4 are lysates from duplicate transient transfection of MT-2 (4μg ml^{-1} and 2μg ml^{-1} respectively), lanes 5 and 6 are duplicate transient transfection of MT-3 (4μg ml^{-1} and 2μg ml^{-1} respectively) and lane 7 is a transient transfection of MT-1 (1μg ml^{-1}) for comparison.

Expression of both the immature and mature tagged c-*fms* isoforms of the Y708F and Y723F mutants was detected in lysate from HEK cells 48 hours post transfection (Fig. 9.6.1., lanes 3 to 6). Although at 48 hours both receptor isoforms are detected, the predominant band corresponds to the immature isoform (135kDa). Expression of both mutant c-*fms* are comparable to the wild-type c-*fms* construct which is also present predominantly in an immature form (Fig. 9.6.1., lane 7). These data suggest that the

that the single amino acid substitutions at the tyrosine residues involved in PI 3-kinase binding do not affect the processing of the immature isoform to the mature form.

9.7.1. Immunofluorescent staining of HEK 293 cells transiently expression MT-2 and MT-3

Confirmation of the cellular location of the Y708F and Y723F mutation containing c-*fms* constructs in HEK cells was carried out by immunofluorescent staining of transiently transfected HEK cells, 24 hours post-transfection (Fig. 9.7.1. and 9.7.2.). Examination of staining was carried out by digital confocal microscopy.

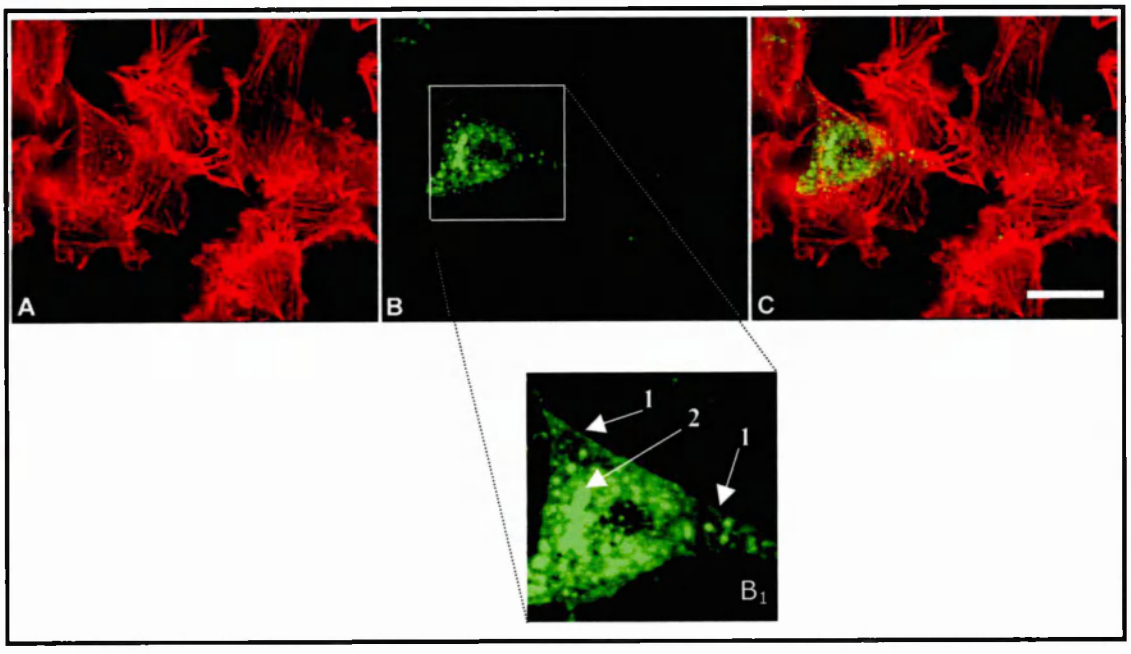


Fig. 9.7.1. Immunofluorescent analysis of MT-2 expression in HEK cells. Transiently transfected HEK cells were probed with an anti-*myc* epitope antibody and stained with an Alexa^{488}-secondary antibody. Panel A is an actin stain, panels B is an anti-*myc* epitope stain at 48 hours post-transfection. Panel C is a composite photomicrograph which indicates positive and negatively stained cells. Panel B_1 is an enlargement of the area marked by a white box in panel B. Images were digitally recorded on an fluorescence inverted microscope and analysed with digital image deconvolution software (OpenLab 1.7.8). White arrows indicate plasma membrane (1) and cytosolic (2) staining. Bar indicates 10μm.

At 24 hours post-transfection HEK cells transfected with the Y708F c-*fms* mutant contained high levels of protein expression, characterised by considerable cytosolic

and plasma membrane staining (Fig. 9.7.1.). Enlargement B_1 in Fig. 9.7.1. demonstrates clearly both plasma membrane staining (Fig. 9.7.1., arrow 1) as well as discrete pockets of cytosolic staining, which are localised in a juxtanuclear region (Fig. 9.7.1., arrow 2). This suggests that the Y708F mutant tagged c-*fms* expression construct is capable of plasma membrane localisation as observed for the wild-type, tagged c-*fms* and for endogenous c-*fms*.

The cellular localisation of the tagged c-*fms* construct containing the Y723F mutation appeared similar to wild-type tagged c-*fms* and to the Y723F mutant. Immuno-fluorescent staining and was characterised by a similar plasma membrane (Fig. 9.7.2., arrow 1) and cytosolic (Fig. 9.7.2., arrow 2) staining pattern to that of the Y708F mutant. This suggests that both the Y708F and Y723F mutant tagged c-*fms* expression constructs are expressed in HEK cells in the anticipated manner and are found expressed at the plasma membrane in transient, 24 hour transfections.

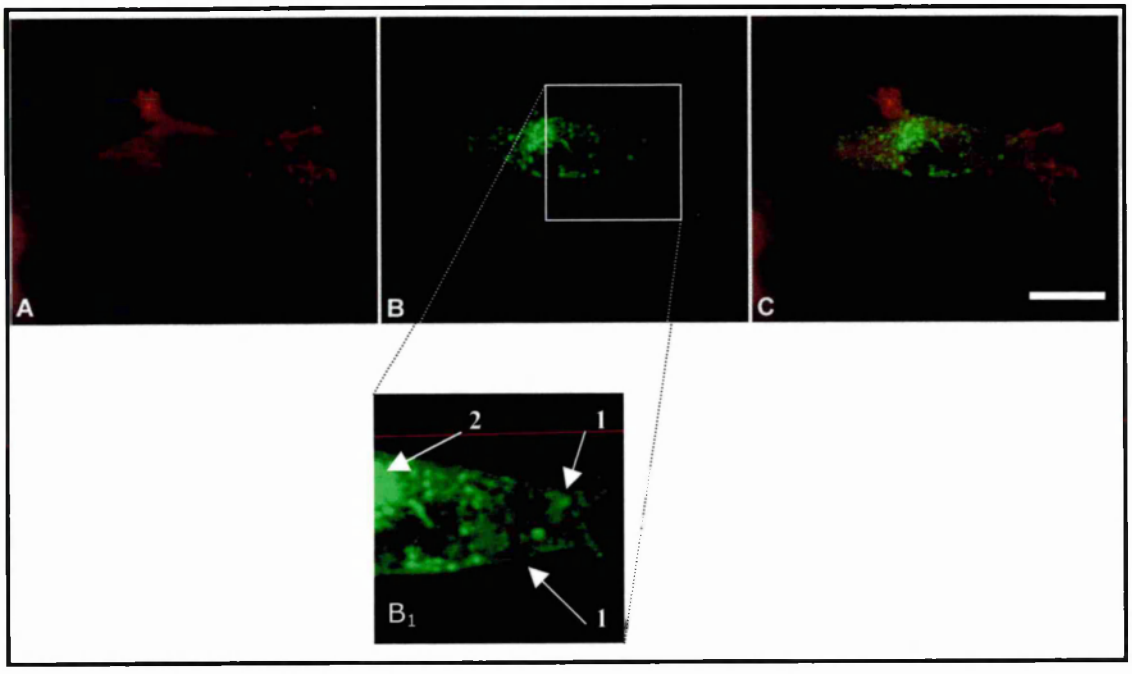


Fig. 9.7.2. Immunofluorescent analysis of MT-3 expression in HEK cells. Transiently transfected HEK cells were probed with an anti-*myc* epitope antibody and stained with an Alexa^{488}-secondary antibody. Panel A is an actin stain, panels B is an anti-*myc* epitope stain at 24 hours post-transfection. Panel C is a composite photomicrograph which indicates positive and negatively stained cells. Panel B_1 is an enlargement of the area marked by a white box in panel B. Images were digitally recorded on an fluorescence inverted microscope and analysed with digital image deconvolution software (OpenLab 1.7.8). White arrows indicate plasma membrane (1) and cytosolic (2) staining. Bar indicates 10μm.

9.8.0. Discussion

Since transfection of macrophage cell lines has not been extensively reported in the present literature, it was necessary to determine the optimum method for BAC1.2F5 transfection. Characterisation of BAC1.2F5 transfection with an eGFP containing vector suggested that transfection and expression of exogenous protein in macrophages was possible. Protein expression was detected in reasonably high numbers of cells (26%) transfected with SuperFect™ reagent, as determined by FACS analysis. This indicated that macrophages were suitable for transfection studies and therefore the c-*fms* construct, MT-1 was transfected into BAC1.2F5 cells. However, expression of this protein was not detected, either by western blot analysis or indirect immunofluorescence. FACS analysis has been demonstrated to be a very sensitive detection method for fluorescently labelled cells and it was possible that FACS detected very low levels of protein expression that were below the sensitivity level for western blot or fluorescence microscopy. Indeed analysis of eGFP-transfected BAC1.2F5 cells showed no discernible difference to untransfected BAC1.2F5 controls (data not shown). This is the most likely explanation for the inability to detect tagged c-*fms*. It has been reported that activation of haematopoietic cells is required for expression of exogenous proteins regulated by the CMV promoter, but activation of BAC1.2F5 cells with PMA did not facilitate tagged c-*fms* detection. At this point it was necessary to demonstrate that MT-1 was capable of coding for and expressing a full length protein and this was confirmed by *in vitro* translation. In either rabbit reticulocyte or wheatgerm agglutinin systems a single, specific protein product of approximately 135kDa was translated from MT-1 (Fig. 9.3.1.). The larger product was predicted from sequence data and the observed immature human c-*fms* protein product and confirmed that the c-*fms* gene was translated and most likely contained both gene specific and *myc*/His tag epitopes.

To confirm that the MT-1 construct did express a protein product detectable by western blot or immunofluorescent analysis, MT-1 had to be expressed in a suitable mammalian cell line. The HEK 293 cell line was chosen because they have routinely demonstrated high transfectability and protein expression. Analysis of whole cell lysate from HEK cells transfected with MT-1 with an anti-*myc* epitope antibody demonstrated that HEK cells the 135kDa and 155kDa isoforms of tagged c-*fms*, at high levels. These bands corresponded to the immature and mature receptor isoforms observed in murine and human cells, although their migration at a higher M_r than normal is due to the addition of the C-terminal tags. However, the expression profile was found to change over time and after 72 hours the mature receptor form was almost absent, but the immature receptor band was unchanged. This suggested that initially c-*fms* is expressed and post-translationally modified as expected and exists as a mature and immature receptor. However, after initial expression, the immature receptor is not modified and remains as an immature isoform. Why this occurs remains unclear, but it is possible that overexpression of this protein in HEK 293 cells affects the expression of other cellular components such that after subsequent rounds of de novo protein synthesis the tagged c-*fms* molecules are selectively not processed or trafficked to the plasma membrane. This may also affect expression of other plasma membrane localised receptors however this was not investigated. C-*fms* was successfully immunoprecipitated from HEK cell lysate with the anti-*myc* epitope antibody which demonstrated that the mature receptor can be purified from cell lysate and may facilitate characterisation of receptor associated proteins.

Successful detection of tagged c-*fms* by western blot analysis, although capable of demonstrating expression, could not reveal the subcellular localisation of tagged c-*fms* in HEK cells. This was accomplished instead by indirect immunofluorescent analysis.

MT-1 expressed in HEK cells was detected by both c-*fms* and *myc* tag specific antibodies after 24 hours (Fig. 9.5.1.). Transiently transfected HEK cells showed plasma membrane and cytosolic staining after 24 hours (Fig. 9.5.1.). At higher magnification the plasma membrane localisation is more obvious and distinct pockets of staining can also be observed in the cytosol (Fig. 9.5.2.). However after 72 hours c-*fms* appears to be localised to a predominantly cytosolic compartment, in a juxtanuclear location which may correspond to the golgi complex (Fig. 9.5.3.). This difference in subcellular localisation, when considered together with the western blot data, suggests that cytosolic staining present in what may be the golgi complex may correspond to the immature c-*fms* isoform. Therefore the plasma membrane staining observed at 24 hours may be due to the correct processing and targeting of the mature receptor isoform.

Together these data confirm that the MT-1, tagged human c-*fms* construct is capable of full length expression in mammalian cells, recognised by both gene–specific and *myc*-tag antibodies. However, the expression of mature protein does appear to be limited to the first 24 hours following transfection in HEK cells and therefore HEK cells may not be a suitable cell line for selection of stable expression. A caveat to this is that c-*fms* is continuously expressed as an immature protein and it may be possible to up-regulate expression of the mature receptor, although this was not investigated..

To validate the expression profile of c-*fms* constructs containing mutations, two representative constructs, MT-2 and MT-3, were characterised by western blot analysis and indirect immunofluorescence similar to MT-1. Both receptor mutants express full-length c-*fms* characterised by detection of both mature and immature isoforms in whole cell lysate from HEK cells, 48 hours after transfection (Fig. 9.6.1.).

However, the predominant form at this time point appears to be the immature form, comparable to HEK cells transfected with the wild-type MT-1 construct (Fig. 9.6.1.). Expression of MT-2 and MT-3 was also characterised by indirect immunofluorescence (Fig. 9.7.1. and 9.7.2.). Expression of either construct appears to be localised to the plasma membrane and the cytosol (Figs. 9.7.1. and 9.7.2.). This localisation profile was similar to the wild-type tagged c-*fms* and demonstrated that mutation of the PI 3-kinase binding sites did not affect the cellular targeting or expression levels in transiently transfected HEK cells.

BAC1.2F5 cells have proved notoriously difficult to transfect and detect expression of exogenous protein. However, these tagged c-*fms* constructs have now been characterised for expression and detection and now provide tools that will facilitate investigation of c-*fms* signalling. The epitope tags present on each construct, with the exception of WT-1, allow the detection of protein expression and distinguish exogenous from endogenous receptor expression. Mutant receptor constructs will allow characterisation of M-CSF stimulated responses where PI 3-kinase association with c-*fms* is required or not as the case may be. Finally, epitope tags will also facilitate precipitation and co-precipitation studies on c-*fms*, and, in conjunction with the mutant receptor constructs, will allow the characterisation of c-*fms* associated signalling molecules and complexes, particularly PI 3-kinase.

Chapter 10

General Discussion

10.0.0. General discussion

M-CSF is an extremely important mediator of macrophage function, involved in the activation of various responses including phagocytosis, enhancement of antibody-dependent cell-mediated cytotoxicity and tumoricidal activity (Lewis, 1992). In addition M-CSF stimulates macrophage survival and proliferation and actively recruits macrophages and monocytes. The M-CSF-deficient mouse knock-out has taught us a substantial amount in general about the various other functions of this growth factor but been less useful for the investigation of M-CSF stimulated cellular responses in macrophages. Analysis of aberrant activation of macrophage responses by M-CSF is relevant to the treatment of diseases such as rheumatoid arthritis, where there is excess M-CSF in rheumatoid joints and macrophage activation is undesirable. Thus the elucidation of M-CSF-induced signalling pathways has both scientific and pathological importance.

To date the mechanisms by which c-*fms* signalling is mediated have not been completely defined. Identification of c-*fms* as a member of the class III growth factor receptor family has actually added to the confusion surrounding c-*fms* signalling since it has led many researchers to draw analogies between its signalling mechanisms and the other family member, the PDGF receptor. This has proven to be inaccurate and many differences between these structurally related receptors are now evident. Throughout the published literature it has become increasingly apparent that exogenous expression studies, although informative, can also be very misleading. Expression of c-*fms* in fibroblasts or epithelial cells has demonstrated associations between c-*fms* and signalling molecules or cell responses which do not appear to exist in macrophages. Therefore c-*fms* expression studies need to be interpreted carefully, always bearing in mind that signalling pathways in fibroblasts do not always mimic

those found in macrophages. C-*fms* is probably not the only molecule that does not couple correctly to downstream mediators in different cell types which is also observed for the PDGF receptor (see chapter 1.6.0.).

The main aim of this thesis was to investigate how PI 3-kinase is involved in c-*fms* signalling. PI 3-kinase is a lipid kinase that produces membrane-bound second messengers that activate various downstream signalling pathways and responses in cells. Investigation of the involvement of PI 3-kinase in c-*fms* signalling, if not complex enough, is further compounded because PI 3-kinase is an enzyme composed of two subunits which also have various subunit isoforms. This added complexity means that c-*fms* has the ability to couple with more than one PI 3-kinase species in macrophages and this may introduce subtle differences that are not or cannot be easily replicated in common cell lines used for expression studies.

Therefore it is difficult to study the complex signalling pathways of c-*fms* without suitable cell models. Various cell lines have been used to investigate c-*fms* signalling, however the BAC1.2F5 murine cell line has provided researchers with a macrophage-like cell line that is dependent on M-CSF for survival and proliferation (Morgan et al., 1987), characteristics found in primary macrophages and monocytes. This cell line has been well characterised with respect to M-CSF stimulated responses such as mitogenesis and morphology, but has not been used to investigate c-*fms* signalling via PI 3-kinase.

In this thesis I have demonstrated;

1. Expression of p85α/p110α in BAC1.2F5 cells.
2. M-CSF stimulation of PI 3-kinase activity.

3. The M-CSF stimulated PI 3-kinase activity is not required for the proliferative signal in BAC1.2F5 cells.
4. PI 3-kinase activity is required for M-CSF mediated cell survival.
5. M-CSF mediates BAC1.2F5 cell survival in concert with LPA and at least one other factor or factors in foetal calf serum.
6. Transport of c-*fms*-containing endocytic vesicles proceeds via a PI 3-kinase-dependent pathway.
7. M-CSF stimulated reorganisation of the actin cytoskeleton requires PI 3-kinase activity.

In addition I have also produced a series of novel human c-*fms* expression constructs containing key amino acid substitutions for the investigation of PI 3-kinase binding to the activated receptor and have demonstrated their expression in epithelial cells.

10.1.0. PI 3-kinase expression

I have demonstrated that BAC1.2F5 cells express the p85α/p110α PI 3-kinase isoforms, representing the class 1a PI 3-kinase activity. Although the p110γ isoform was also present it is unlikely to be activated M-CSF as this has been shown to be activated by G-protein linked receptors. Thus the major M-CSF responsive PI 3-kinase activity is likely to be effected by p85α/p110α heterodimers. The p85α/p110α PI 3-kinase has also been demonstrated to be stimulated by M-CSF and was sensitive to wortmannin and LY294002 when used at concentrations at their respective IC_{50} or at concentrations previously reported to only inhibit PI 3-kinase activity. This stimulation of activity is consistent with data showing that the p85α/p110α can be co-immunoprecipitated with c-*fms* from BAC1.2F5 cells (Kanagasundaram et al., 1996).

Based on these observations the various M-CSF stimulated responses in BAC1.2F5 cells were characterised using PI 3-kinase inhibitors.

10.2.0. PI 3-kinase mediated survival and proliferation

I have demonstrated that inhibition of PI 3-kinase activity does not block the M-CSF stimulated c-*fms*-mediated proliferation signal in BAC1.2F5 cells. These data are similar to observations made by Kanagasundaram et al, (1996) who found no role for PI 3-kinase activity in M-CSF-mediated stimulation of proliferation in BAC1.2F5 macrophages. However, the proliferation assay data presented in chapter 5 was expressed as a percentage of the total cell population, and did not reflect the requirement for PI 3-kinase in M-CSF stimulated survival.

In cell density assays carried out over 4 days M-CSF induced BAC1.2F5 cell doubling every 24 hours, and this required the presence of foetal calf serum, which suggested that another factor or factors, present in serum, were required for cell proliferation. It had recently been demonstrated that peritoneal macrophages required LPA for survival and so a requirement for LPA in BAC1.2F5 cell growth was investigated by using delipidated serum and exogenous LPA. This revealed that delipidated serum contains a factor or factors other than LPA which are required for M-CSF stimulated increases in BAC1.2F5 cell population. Investigation of the effect of LY294002 on M-CSF stimulated increases in cell population confirmed PI 3-kinase was required for population increase over 4 days. Therefore although PI 3-kinase was not required for the proliferative signal it was required for cell survival and the observed data may represent a balance between cell death and cell proliferation, which are controlled by two different signalling pathways, one

involving PKB activation and the other probably requiring PI 3-kinase independent ras activation, respectively and the net effects of these are summarised in Fig. 10.2.1.

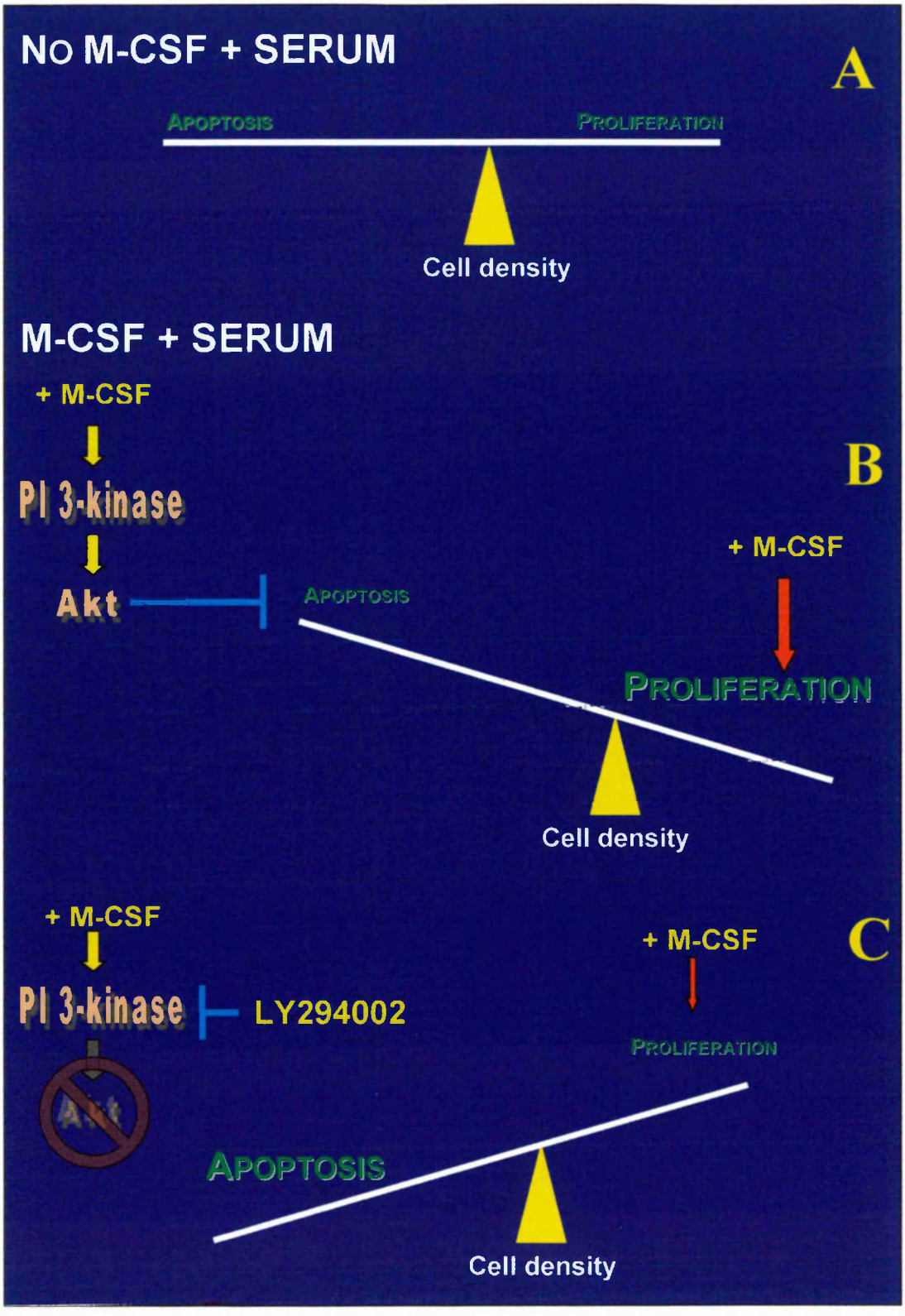


Fig. 10.2.1. Summary of data from chapter 5 illustrating PI 3-kinase-mediated protection against apoptosis and its effects on cell growth in BAC1.2F5 macrophages.

Fig. 10.2.1. shows a graphical summary and interpretation of the data presented in chapter 5. The investigation of BAC1.2F5 apoptosis revealed that there is a basal rate of apoptosis in BAC1.2F5 cell of 2-4%, which increased upon M-CSF withdrawal. I presented data that shows addition of PI 3-kinase inhibitor, LY294002, induced >65% apoptosis in M-CSF stimulated cells which confirmed that protection against apoptosis in BAC1.2F5 cells conferred by M-CSF is dependent on PI 3-kinase activation (Fig. 10.2.1.). This data explains the reduction in cell population observed in the cell density assays.

In addition it has been shown that factors in serum are required for increases in cell density. In corollary experiments it was demonstrated that serum contains multiple factors which protect BAC1.2F5 cells against apoptosis. One such factor was likely to be LPA but reconstitution of LPA alone was not sufficient for the protective effects seen in full serum. Addition of delipidated serum and LPA with M-CSF indicated that at least three factors, including M-CSF and LPA, act synergistically to confer protection against apoptosis in BAC1.2F5 cells.

Thus the model proposed for M-CSF stimulated survival and proliferation in BAC1.2F5 cells is that in the absence of M-CSF there is a balance between the apoptosis and proliferation which also depends on serum factors as well as M-CSF (Fig. 10.2.1., A). However, in the absence of M-CSF there is an eventual swing towards apoptosis. In the presence of M-CSF there are two signals, one which stimulates cell proliferation and is independent of PI 3-kinase activity and a second which protects against apoptosis in a PI 3-kinase-dependent manner, possibly through Akt/PKB (Fig. 10.2.1., B). If PI 3-kinase activity is inhibited in M-CSF stimulated cells, although the proliferative signal is still present the pathway leading to protection

against apoptosis is blocked and therefore cells enter into apoptosis (Fig. 10.2.1., C). Therefore the signalling pathway leading to apoptosis is the control mechanism for both cell survival and proliferation, for net increases in cell density can only occur if there is a protective anti-apoptotic signal, mediated by PI 3-kinase.

10.3.0. PI 3-kinase and c-*fms* trafficking

In BAC1.2F5 cells incubated with either LY294002 or wortmannin, although some cell spreading and increased membrane activity was observed this was substantially less than in untreated cells. More interestingly, formation of phase-light vesicles around the periphery of the cell proceeded as normal, the trafficking of these newly formed vesicles required PI 3-kinase activity . These data confirmed that PI 3-kinase activity was required for transportation of endocytic vesicles to sites of lysosomal degradation.

More detailed analysis of c-*fms* internalisation revealed that the receptor is internalised to small endocytic vesicles characterised by distinct punctate staining pattern around the cell periphery which are trafficked towards the nucleus. These endocytic vesicles then appear to coalesce to form substantially larger endocytic vesicles clustered around the nucleus. Although these endocytic vesicles remain for over an hour after internalisation the c-*fms* staining is almost completely absent by 30 minutes, which is consistent with the observed time-course of degradation for M-CSF (Boocock et al., 1989).

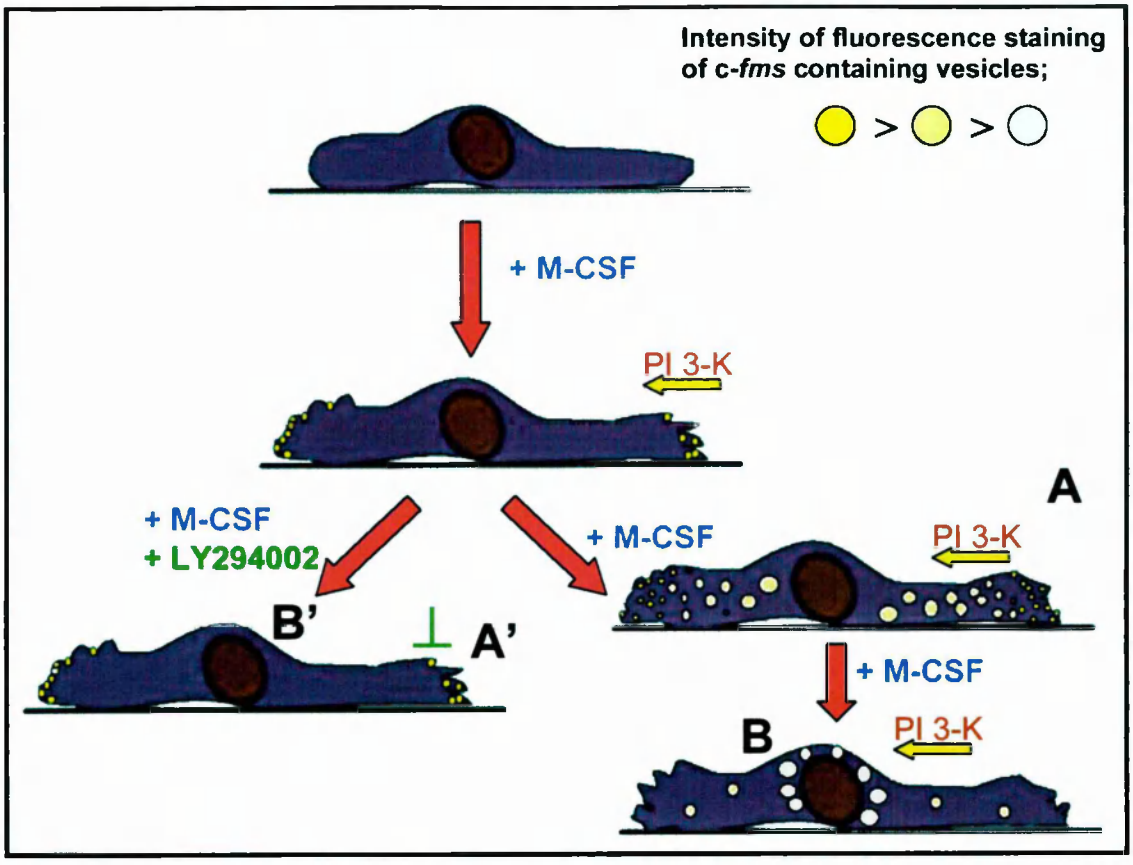


Fig. 10.3.1. Model for the M-CSF-stimulated formation and transport of receptor containing endocytic vesicles in BAC1.2F5 macrophages. Vesicle shading represents the proportion of c-fms staining on the vesicle membrane.

Therefore a model of c-*fms* internalisation can be proposed in which internalisation of receptor proceeds normally (Fig. 10.3.1., A) and is not affected by inhibition of PI 3-kinase activity resulting in receptor degradation (Fig. 10.3.1. B). However, transport of c-*fms*-containing vesicles towards the nucleus is impaired (Fig. 10.3.1., A') and this also prevents receptor degradation (Fig. 10.3.1., B'). This suggests that PI 3-kinase activity is required during vesicle transport probably at a post-endocytic step between the juxtamembrane and the lysosomal compartments. These observations are consistent with a similar observation in fibroblasts where internalisation of the PDGFr is blocked at a juxtamembrane site in the presence of wortmannin (Joly et al., 1995).

10.4.0. PI 3-kinase and the actin cytoskeleton

The actin cytoskeleton is an integral part of many macrophage responses including membrane activity, cell motility and vesicular transport, and BAC1.2F5 cells have been well characterised in this respect (Allen et al., 1997). In some early studies on BAC1.2F5 cells it was observed that they responded to M-CSF with dramatic changes in cell shape and morphology (Boocock et al., 1989). The data presented in this thesis suggests that PI 3-kinase activity is required for certain aspects of actin reorganisation possibly mediated by rac and rho and it is proposed that the effects on the actin cytoskeleton may account for the effects of PI 3-kinase inhibitors on vesicle transport.

In conjunction with vesicle formation and transport there are significant M-CSF stimulated changes in the actin cytoskeleton, characterised by the formation of filopodia, membrane ruffles and fine actin cables. These actin rearrangements occur rapidly and the actin cables persist for at least 30 minutes, similar to the time-course of receptor internalisation. The localisation of c-*fms* containing internalised vesicles and the newly formed actin cables suggests that the actin cytoskeleton is employed during vesicular transport, which has been reported for receptor mediated endocytosis into clathrin-coated pits. Inhibition of PI 3-kinase activation had quite striking effects on vesicle transport and rearrangement of the actin cytoskeleton. LY294002 treated BAC1.2F5 cells possessed retraction fibres with reduced membrane ruffle formation and little or no actin cables. In addition, treatment of M-CSF stimulated cells blocked transport of newly formed endocytic vesicles from the plasma membrane where they remained as small punctate structures.

The absence of actin cables coincided with aberrant vesicle transport and our conclusion is that reorganisation of the actin cytoskeleton is a prerequisite for correct

vesicle transport in BAC1.2F5 macrophages. The reorganisation of the actin cytoskeleton appears to be mediated by PI 3-kinase activation, and although this may explain the aberrant vesicle transport, it is possible that PI 3-kinase products are also directly involved in vesicle trafficking.

In light of the data presented in this thesis on the requirement for PI 3-kinase activity for the correct reorganisation of the actin cytoskeleton, the model proposed in section 10.3.0. can be modified further to include the organisation of the actin cytoskeleton (Fig. 10.4.1.). Stimulation of BAC1.2F5 cells stimulates the rapid formation of c-*fms*-containing vesicles after only 5 minutes and this is independent of PI 3-kinase (Fig. 10.4.1. A and B, 5'). Concomitantly with vesicle formation is the appearance of fine actin cables organised longitudinal to the direction of cell polarisation (Fig. 10.4.1. A, 5'). This process is dependent on PI 3-kinase activity and F-actin staining in cells where PI 3-kinase activity is inhibited is punctate and present only on the ventral surface of the cell (Fig. 10.4.1. B, 5'). After 10 minutes the c-*fms*-containing vesicles have migrated towards the nucleus (Fig. 10.4.1. A, 10') but this is absent in LY294002 treated cells where vesicles remain at the plasma membrane (Fig. 10.4.1. B, 10'). C-*fms*-containing vesicles tend to co-localise with the fine actin cables and it is possible that these structures mediate vesicle transport. After 30 minutes large phase light vesicles are present around the periphery of the nucleus but do not contain c-*fms* (Fig. 10.4.1. A, 30'). In cells treated with LY294002 vesicles still remain at the plasma membrane and still contain high levels of c-*fms* (Fig. 10.4.1. B, 30'). In human macrophages it has been demonstrated that inhibition of actin microfilament assembly by cytocholasin B blocks FcR-mediated phagocytosis in human macrophages (Newman et al, 1991). Therefore PI 3-kinase activity is required for the reorganisation of the actin cytoskeleton and this may be required for the correct

trafficking of internalised receptor, via clathrin coated vesicles, to downstream endocytic and lysosomal compartments for eventual degradation.

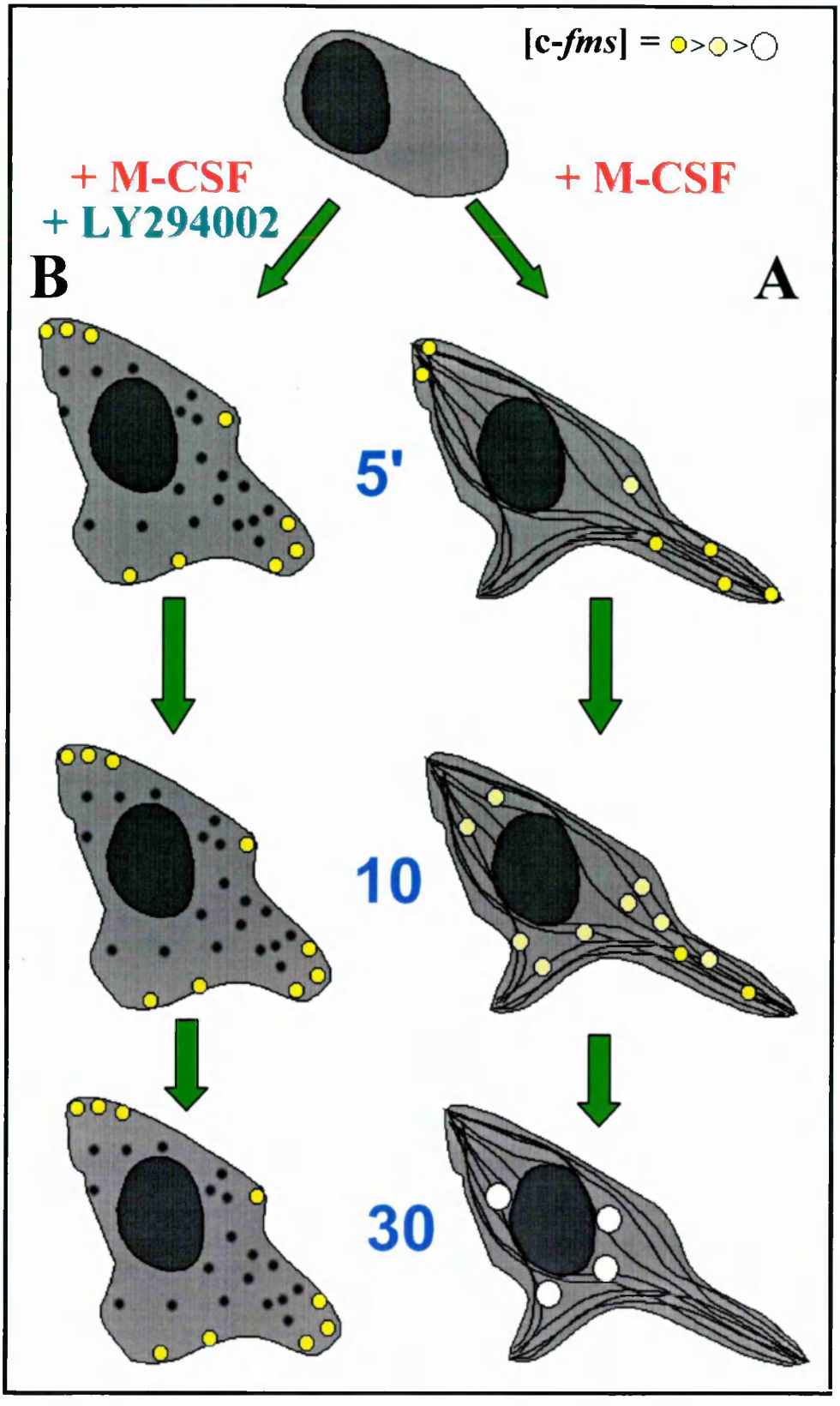


Fig. 10.4.1. Model for c-*fms* internalisation and trafficking in BAC1.2F5 cells which relies on the correct reorganisation of the actin cytoskeleton in a PI 3-kinase-dependent manner.

10.5.0. Production of c-*fms* expression constructs

The use of PI 3-kinase inhibitors has demonstrated that PI 3-kinase activation is an integral part of at least two signalling pathways downstream of c-*fms* (Fig. 10.5.1.). These are (a) the protection against apoptosis which possibly proceeds via Akt and which also has profound effects on cell proliferation and (b) the correctly regulated reorganisation of the actin cytoskeleton which is essential for cell motility and structural integrity, and may also account for the incorrect transport of endocytic vesicles. A summary of these PI 3-kinase mediated processes is depicted in Fig. 10.5.1.

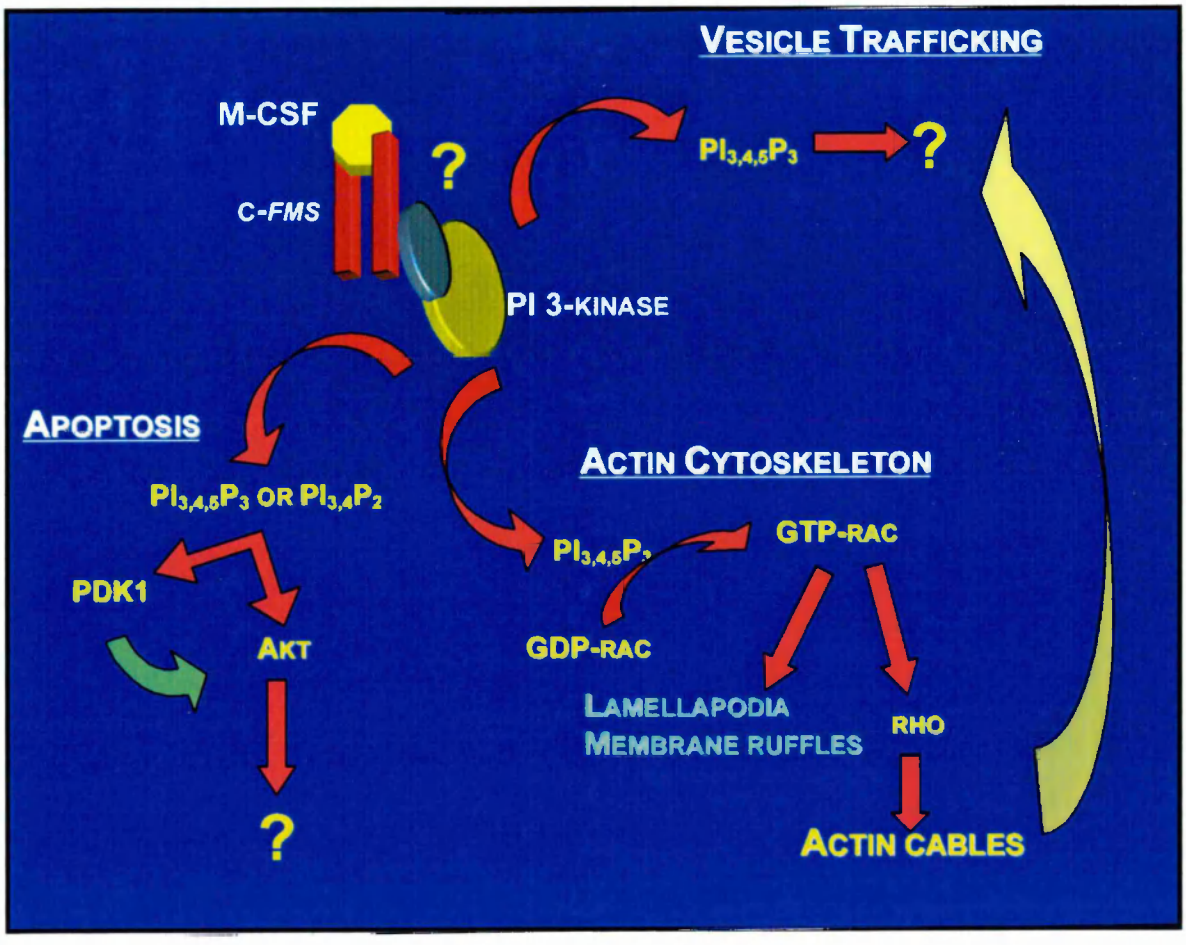


Fig. 10.5.1. Summary of the PI 3-kinase dependent pathways in BAC1.2F5 macrophages.

Although the available PI 3-kinase inhibitors have revealed a substantial amount of information regarding PI 3-kinase mediated, M-CSF signalling in this macrophage

cell model have two drawbacks. Firstly, these inhibitors cannot be used to differentiate between PI 3-kinase activities from class 1 or class 3 PI 3-kinases, and secondly we cannot obtain any information regarding the association of PI 3-kinase with c-*fms*, whether that is direct or indirect via adapter molecules. Therefore new tools for the analysis of PI 3-kinase-c-*fms* interactions were required.

The approach taken in this thesis was to produce a series of novel human c-*fms* constructs containing mutations at key residues in the KI domain, involved or implicated in PI 3-kinase binding. These constructs would possess convenient epitope tags to facilitate identification and purification. The presence of an epitope tag to facilitate purification was important since it was intended that these constructs be expressed in BAC1.2F5 macrophages, hence easy identification was necessary to differentiate between endogenous and exogenous c-*fms* expression. Although all the human c-*fms* constructs were made successfully, it became apparent that difficulties associated with expression studies in haematopoietic lineage cells would prevent the intended goal. I have demonstrated that transfection of BAC1.2F5 macrophages can be accomplished. However, it is apparent that this does not equate to expression of specific heterologous proteins and in particular it appears that expression constructs containing the CMV promoter are not suitable for expression in these cells. As an alternative, three of the c-*fms* constructs were expressed in epithelial cells to confirm that, in practice, the features of these constructs were valid. Indeed, high levels of transient expression were detected by both western blot and immunofluorescent analysis by both gene and epitope-tag specific antibodies. However what became evident was that in HEK 293 cells at least, c-*fms* expression profiles change over time. This was characterised by a loss of expression of the mature receptor isoform and loss of membrane expressed receptor. These data reinforce the difficulties associated with

exogenous expression studies in non-native cell lines, in this instance loss of functional c-*fms* expression, and which leads to a need for caution when interpreting data.

10.6.0. Suggestions for future work

In this thesis I have defined the key areas in which PI 3-kinase activity plays a role in M-CSF signalling. However this area of research is far from being complete and requires further study.

Central to defining the PI 3-kinase mediated signalling pathways downstream of c-*fms* is the ability to define how PI 3-kinase is activated by c-*fms*, whether that is by direct interaction via receptor tyrosine phosphorylation sites or indirectly via alternative mechanisms whether that be through adapter molecules or further downstream, away from the receptor complex. This will be facilitated by the use of the receptor constructs generated by this thesis and it is believed that the mutant receptor constructs can be used to determine whether different PI 3-kinase mediated responses are activated via direct interaction with c-*fms*, indirect activation or whether both these mechanisms are involved in activation of different PI 3-kinase mediated responses.

Although the constructs produced from this thesis have had their fidelity confirmed in an epithelial cell line it is not desirable to further investigate c-*fms* signalling in this cellular context. It is more desirable to express these mutant constructs in a cells like BAC1.2F5's. The problems associated with expression of protein may be overcome in a number of ways including; use of an alternative promoter in the construct such as an endogenous haematopoietic promoter, or alternatively transferring to a viral based

infection system such as adenovirus which has proven successful for others. An endogenous promoter may not provide high levels of expression, but this is more desirable than none at all from the present CMV promoter.

Further studies of PI 3-kinase signalling will employ novel ways of interfering with the normal function of specific PI 3-kinase isoforms. The cloning of each PI 3-kinase isoform has provided the raw materials for construction of isoform-specific dominant negative and constitutively active constructs which, when expressed in suitable cell lines will provide better tools for investigation of PI 3-kinase signalling downstream of activated growth factor receptors. Existing p110α PI 3-kinase constructs still have the same drawback as most other expression constructs, that they are regulated by the CMV promoter. Therefore they are unsuitable for use in the cell model required for analysis of c-*fms* signalling.

Investigation of the specific responses in BAC1.2F5 cells mediated by PI 3-kinase activation also requires further investigation. One novel way of molecularly differentiating between the PI 3-kinase products which are important for signalling can be carried out using the recently cloned lipid 5-phosphatase, synaptojanin, which has been demonstrated to hydrolyse $PI_{3,4,5}P_3$ to $PI_{4,5}P_2$. It would be of interest to investigate the effects of synaptojanin overexpression on M-CSF stimulated responses in macrophages. Overexpression of this 5-phosphatase activity should lower $PI_{3,4,5}P_3$ levels, and therefore, if $PI_{3,4,5}P_3$ is involved in cytoskeletal reorganisation and/or vesicle transport then this should result in a reduced response. Conversely, if $PI_{4,5}P_2$ was the key regulator of these responses then synaptojanin overexpression should increase the cell's responses. However, the problems associated with transfection and expression in macrophages would still be significant hurdle. The problems associated

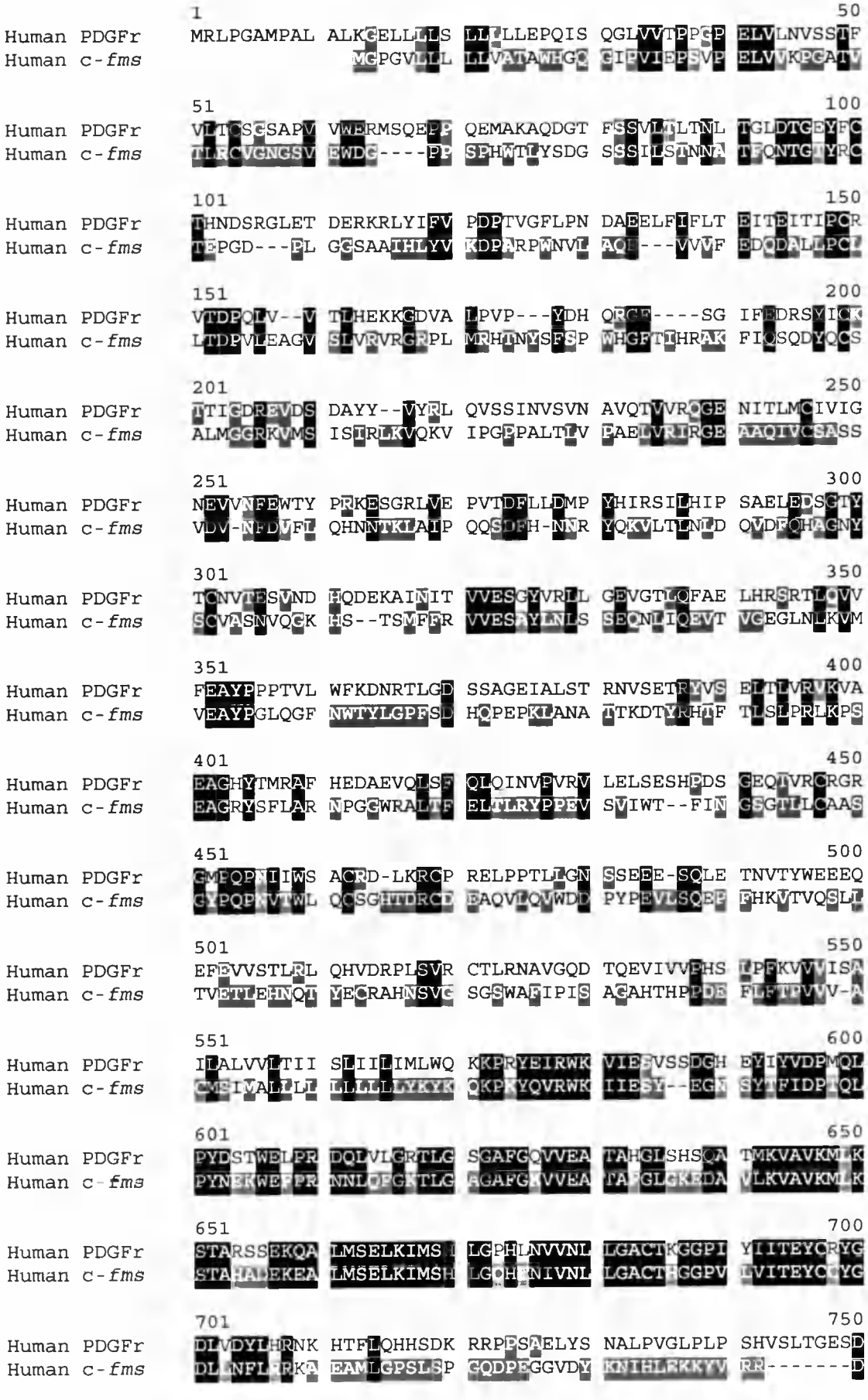
with macrophage transfection may also be overcome using expression constructs containing endogenous mammalian promoters. For example, real-time analysis of actin dynamics can be investigated with an actin-eGFP construct under the control of the actin promoter. Therefore further investigation of receptor-mediated endocytosis with mutant c-*fms* and PI 3-kinase expression constructs with suitable promoters will facilitate expression in haematopoietic cells, such as BAC1.2F5's, and will result in new insights into c-*fms*-mediated responses involving PI 3-kinase activation.

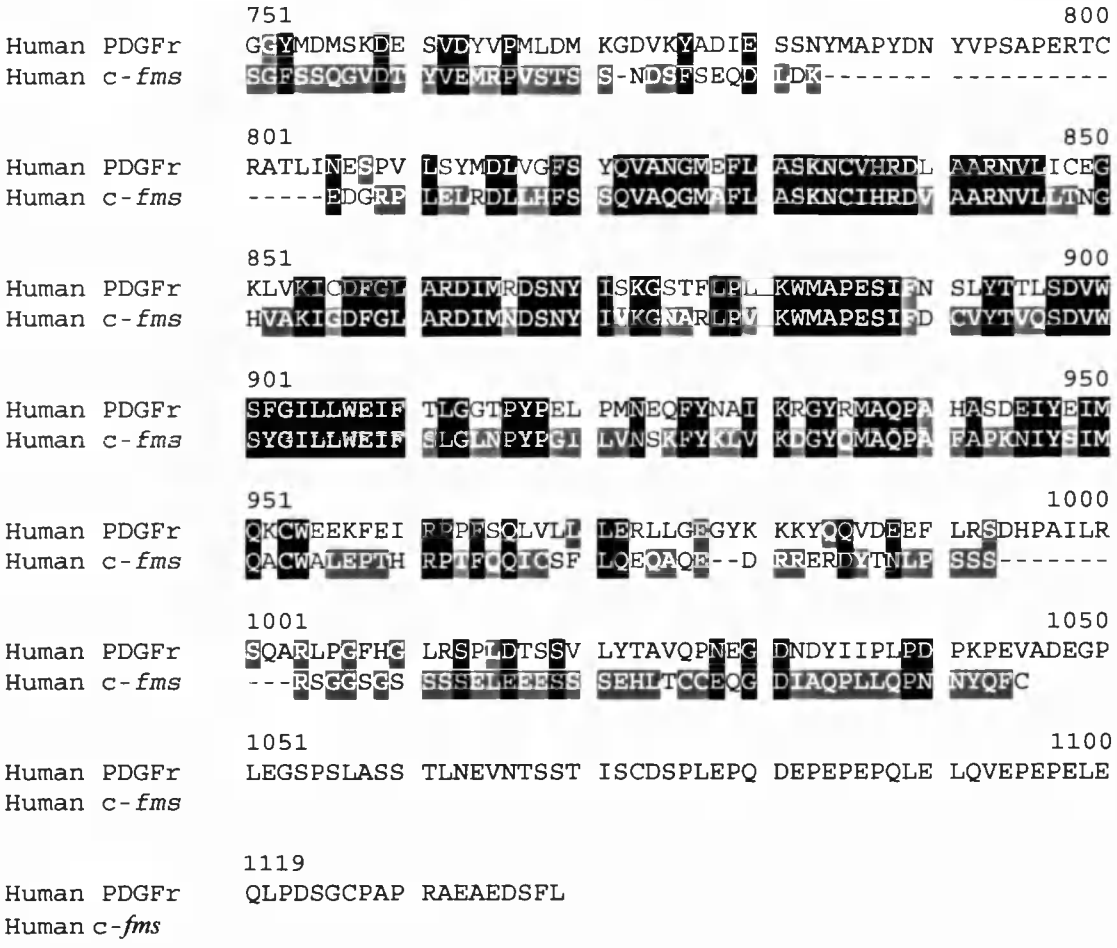
Other novel tools for the analysis of PI 3-kinase signalling may be found in the development of new drugs with specificity for the individual isoforms of PI 3-kinase. A drug based approach however has proved elusive and very little progress has been made towards new novel compounds. The existing drugs that inhibit PI 3-kinase act at sights crucial for enzymatic activity, but as a result are not specific for p110 isoforms due to the very high homology between isoforms. Thus development of new drugs that interfere with p110 interactions with regulatory subunits or other signalling molecules that associate with PI 3-kinase may provide useful tools for the investigation of PI 3-kinase signalling and therefore, in turn, c-*fms* signalling.

Despite the wealth of data on c-*fms* signalling which has been generated over the past thirteen years this area of tyrosine kinase research continues to be extremely active and it appears that it will remain so since there is so much more about c-*fms* signalling that has yet to be elucidated.

Appendix

Multalin version 5.3.3 (Corpet, 1988)
Symbol comparison table: blosum62
Gap weight: 12 Gap length weight: 2




751 800
Human PDGFr GGYMDMSKDE SVDYVPMLDM KGDVKYADIE SSNYMAPYDN YVPSAPERTC
Human c-fms SGFSSQGVDT YVEMRPVSTS S-NDSFSEQD LDK------- ----------
801 850
Human PDGFr RATLINESPV LSYMDLVGFS YQVANGMEFL ASKNCVHRDL AARNVLICEG
Human c-fms -----EDGRP LELRDLLHFS SQVAQGMAFL ASKNCIHRDV AARNVLLTNG
851 900
Human PDGFr KLVKICDFGL ARDIMRDSNY ISKGSTFLPL KWMAPESIFN SLYTTLSDVW
Human c-fms HVAKIGDFGL ARDIMNDSNY IVKGNARLPV KWMAPESIFD CVYTVQSDVW
901 950
Human PDGFr SFGILLWEIF TLGGTPYPEL PMNEQFYNAI KRGYRMAQPA HASDEIYEIM
Human c-fms SYGILLWEIF SLGLNPYPGI LVNSKFYKLV KDGYQMAQPA FAPKNIYSIM
951 1000
Human PDGFr QKCWEEKFEI RPPFSQLVLL LERLLGEGYK KKYQQVDEEF LRSDHPAILR
Human c-fms QACWALEPTH RPTFQQICSF LQEQAQE--D RRERDYTNLP SSS-------
1001 1050
Human PDGFr SQARLPGFHG LRSPLDTSSV LYTAVQPNEG DNDYIIPLPD PKPEVADEGP
Human c-fms ---RSGGCSG SSSELEEESS SEHLTCCEQG DIAQPLLQPN NYQFC
1051 1100
Human PDGFr LEGSPSLASS TLNEVNTSST ISCDSPLEPQ DEPEPEPQLE LQVEPEPELE
Human c-fms
1119
Human PDGFr QLPDSGCPAP RAEAEDSFL
Human c-fms

Cloning Primers

AF-1 5′ Xho I GC=47.8%

5′CCACCTCGAGAAGAAATATGTCC3′

AR-1 3' Hind III GC=46.4%

5′CGAAGCTTGCAGAACTGATAGTTGTTGG3′

AR-2 3′ Hom GC=46.4%

5′CGAAGCTTTCAGCAGAACTGATAGTTGTTGG3′

AR-3 3′ Hom GC=45.2%

5′ACTCCTCAGCAGAACTGATAGTTGTTGG3′

AF-2 5′ Eco RI GC=52.0%

5′GTAGAATTCATAGGCCATGGGCCCA3′

AR-4 3′ Xho I GC=47.8%

5′GGACATATTTCTTCTCGAGGTGG3′

AF-3 5′ Hom. GC=56.5%

5′GCTAGTAGCTGAGAGCTCTGTGC3′

Analysis of full length c-fms gene

SF-1 GC=66.7%

5′GTGCTGGAAGCAGGCGTCTCG3′

SF-2 GC=52.2%

5′GTTTGAGCTCACCCTTCGATACC3′

SF-3 GC=48.0%

5′TGGAAGATCATCGAGAGCTATGAGG3′

SF-4 GC=44.0%

5′ATCCACCTCGAGAAGAAATATGTCC3′

SF-5 GC=42.3%

5′CAGCAAGTTCTATAAACTGGTGAAGG3′

SR-1 GC=61.9%

5′AGCAGACAGGGCAGTAGTGCG3′

SR-2 GC=57.7%

5′TACCACCCGGAAGAACATGGAGGTGG3′

SR-3 GC=57.7%

$^{5'}$ACTGCACTGCAGCCATGTCACGTTGG$^{3'}$

SR-4 GC=51.9%

$^{5'}$AACTCCCACTTCTCGTTGTAAGGCAGC$^{3'}$

SR-5 GC=61.9%

$^{5'}$TGGCTGGAGAAGCCACTGTCC$^{3'}$

SR-6 GC=53.8%

$^{5'}$AAGGAAGGAGCAGATCTGCTGGAAGG$^{3'}$

T7 Promoter GC=40.0%

$^{5'}$TAATACGACTCACTATAGGG$^{3'}$

M13/pUC Reverse GC=43.5%

$^{5'}$AGCGGATAACAATTTCACACAGG$^{3'}$

BGH Reverse GC=55.6%

$^{5'}$TAGAAGGCACAGTCGAGG$^{3'}$

Site-Directed Mutagenesis Primers

Tyr723Phe-1 GC=60.0%

$^{5'}$CCAGGGTGTGGACACCTTTGTGGAAGATGAGGCCTG$^{3'}$

Tyr723Phe-2 GC=60.0%

$^{5'}$CAGGCCTCATCTCCACAAAGGTGTCCAACACCCTGG$^{3'}$

Tyr708Phe-1 GC=55.0%

$^{5'}$CATCCACCTCGAGAAGAAATTTGTCCGCAGGGACAGTGGC$^{3'}$

Tyr708Phe-2 GC=55.0%

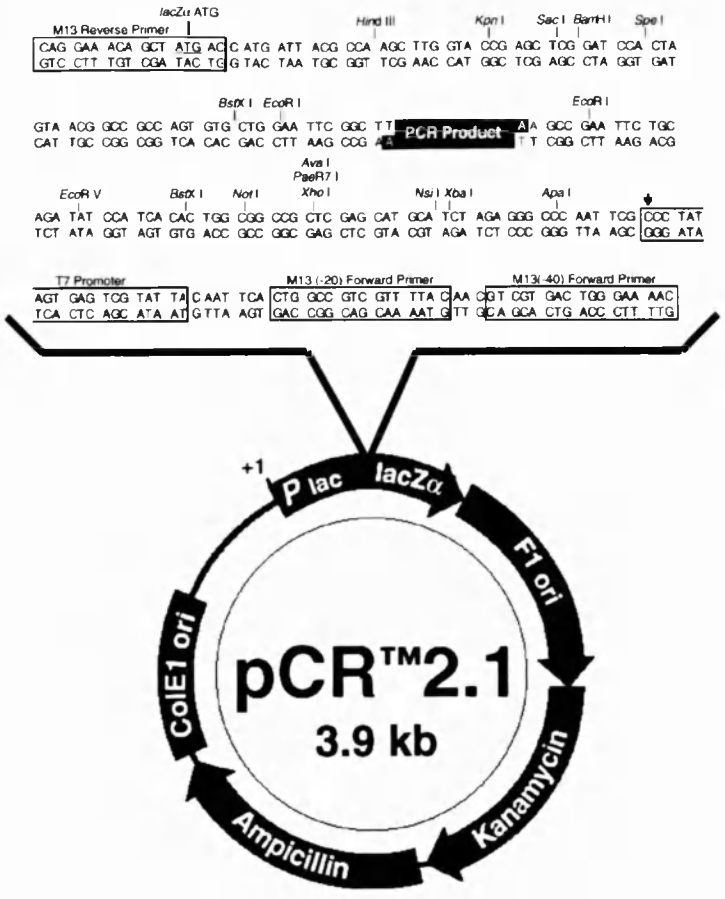
$^{5'}$GCCACTGTCCCTGCGGACAAATTTCTTCTCGAGGTGGATG$^{3'}$

Leu301Ser-1 GC=53.8%

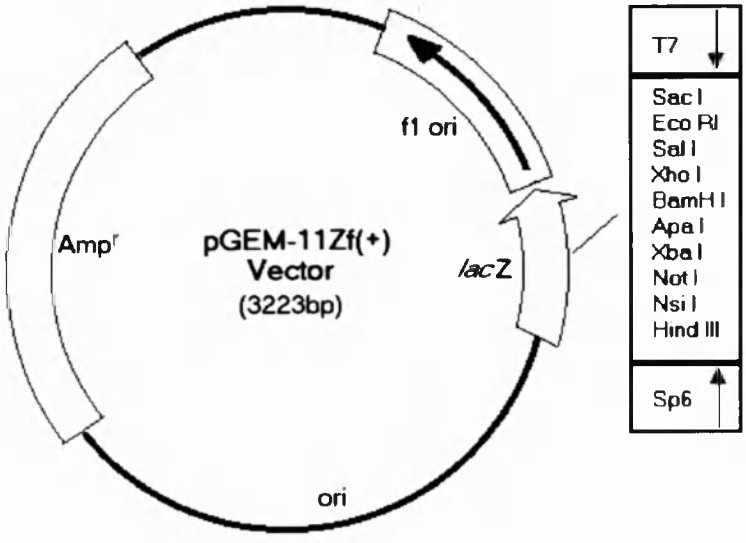
$^{5'}$GAGAGTGCCTACTCGAACTTGAGCTC$^{3'}$

Leu301Ser-2 GC=53.8%

$^{5'}$GAGCTCAAGTTCGAGTAGGCACTCTC$^{3'}$



A. **Diagram of the PCR cloning vector pCR2.1 and a schematic of the multiple cloning site.**



B. **Diagram of the shuttle vector pGEM-11Zf(+) and its multiple cloning site.**

```
                                enhancer region (3' end)
689  CATTGACGTC AATGGGAGTT TGTTTTGGCA CCAAAATCAA CGGGACTTTC CAAAATGTCG

                                    CAAT                              TATA
749  TAACAACTCC GCCCCATTGA CGCAAATGGG CGGTAGGCGT GTACGGTGGG AGGTCTATAT

           3' end of hCMV                putative transcriptional start
809  AAGCAGAGCT CTCTGGCTAA CTAGAGAACC CACTGCTTAC TGGCTTATCG AAATTAATAC

  T7 promoter primer binding site          Nhe I      Pme I      Apa I Xba I   Xho I   Not I
869  GACTCACTAT AGGGAGACCC AAGCTGGCTA GCGTTTAAAC GGGCCCTCTA GACTCGAGCG

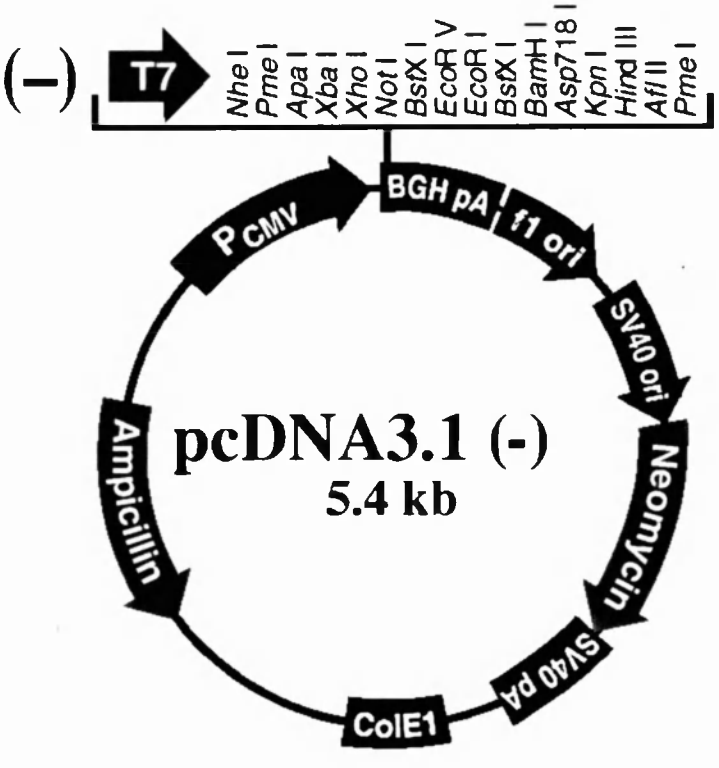
                  BstX I  EcoR V      EcoR I      BstX I            BamH I
929  GCCGCCACTG TGCTGGATAT CTGCAGAATT CCACCACACT GGACTAGTGG ATCCGAGCTC

  Asp718 I  Kpn I  Hind III  Afl II   Pme I                pcDNA3.1/BGH reverse priming site
989  GGTACCAAGC TTAAGTTTAA ACCGCTGATC AGCCTCGACT GTGCCTTCTA GTTGCCAGCC

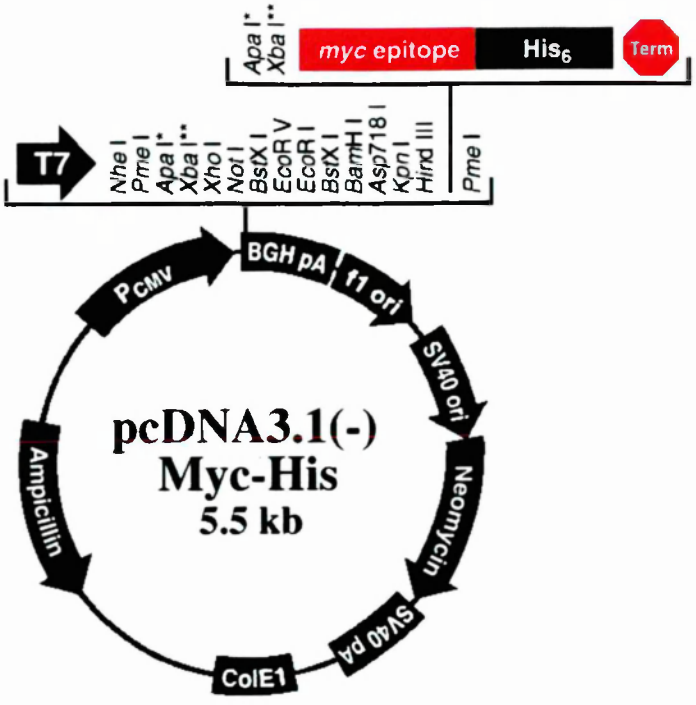
1049 ATCTGTTGTT TGCCCCTCCC CCGTGCCTTC CTTGACCCTG GAAGGTGCCA CTCCCACTGT

           BGH poly (A) site
1109 CCTTTCCTAA TAAAATGAGG AAATTGCATC
```

C. Diagram of the multiple cloning site of pcDNA3.1(-).



D. Diagram of pcDNA3.1(-).



E. Diagram of pcDNA3.1(-)-myc/His.

*The Apa I site is redundant in reading frame A only.

**The Xba I site is redundant in reading frame B only.

```
                T7 promoter/priming site                                  Hind III          Kpn I
861  ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGT TA AGC TTG GTA CCG AGC
                                                    Ser Leu Val Pro Ser

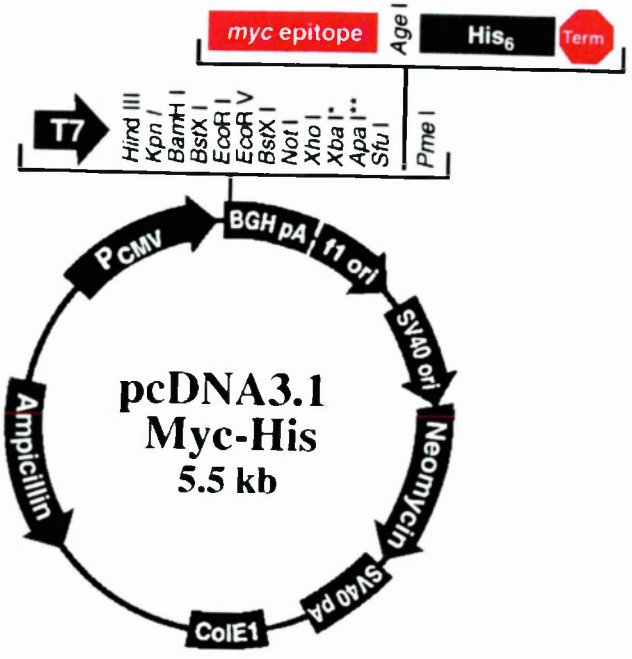
     BamH I                              BstX I  EcoR I              EcoR V          BstX I
918  TCG GAT CCA CTA GTC CAG TGT GGT GGA ATT CTG CAG ATA TCC AGC ACA GTG
     Ser Asp Pro Leu Val Gln Cys Gly Gly Ile Leu Gln Ile Ser Ser Thr Val

     Not I     Xho I    BstE II          Sfu I                    myc epitope
969  GCG GCC GCT CGA GGT CAC CCA TTC GAA CAA AAA CTC ATC TCA GAA GAG GAT
     Ala Ala Ala Arg Gly His Pro Phe Glu Gln Lys Leu Ile Ser Glu Glu Asp

                         Age I          Polyhistidine tag                  Pme I
1020 CTG AAT ATG CAT ACC GGT CAT CAT CAC CAT CAC CAT TGA GTTTAAACCC
     Leu Asn Met His Thr Gly His His His His His His ***

                pcDNA3.1/BGH Reverse priming site
1069 GCTGATCAGC CTCGACTGTG CCTTCTAGTT GC
```

F. Multiple cloning site of pcDNA3.1(+)-myc/His in the C reading frame.



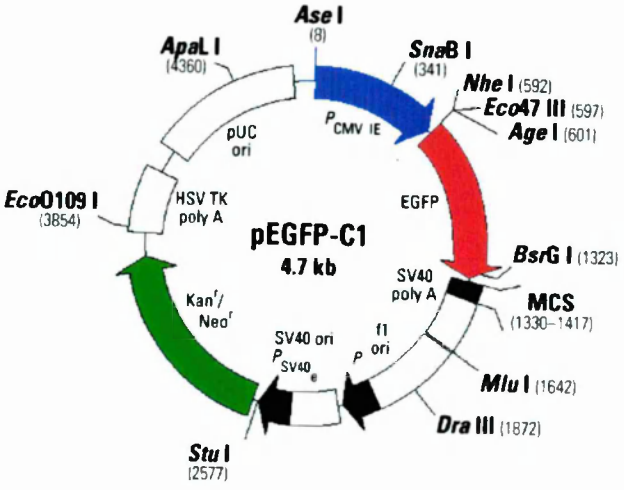
G. Diagram of pcDNA3.1(+)-myc/His.

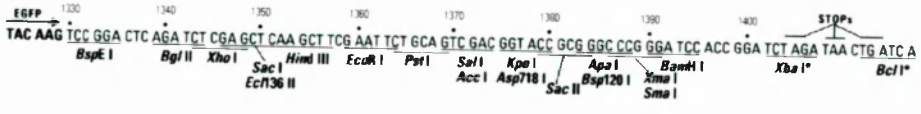
* Unique Sac II site between the Apa I and the Sfu I in reading frame B.

** Unique BstE II site, but no Xba I or Apa I sites in reading frame C.

pEGFP-C1 Vector

GenBank Accession #: U55763





Restriction Map and Multiple Cloning Site (MCS) of pEGFP-C1.

H. Diagram of peGFP-C1.

Multiple alignment of 5' and 3' end of WT-1, Human c-fms in pcDNA3.1(-).

```
Hu c-fms CCTGCGGAGC TAGTAGCTGA GAGCTCTGTG CCCTGGGCAC CTTGCAGCCC  269

WT-1 #1  --------GC TAGTAGCTGA GAGCTCTGTG CCCTGGGCAC CTTGCAGCCC
WT-1 #2  --------GC TAGTAGCTGA GAGCTCTGTG CCCTGGGCAC CTTGCAGCCC

Hu c-fms TGCACCTGCC TGCCACTTCC CCACCGAGGC CATGGGCCCA GGAGTTCTGC  319

WT-1 #1  TGCACCTGCC TGCCACTTCC CCACCGAGGC CATGGGCCCA GGAGTTCTGC
WT-1 #2  TGCACCTGCC TGCCACTTCC CCACCGAGGC CATGGGCCCA GGAGTTCTGC

Hu c-fms TGCTCCTGCT GGTGGCCACA GCTTGGCATG GTCAGGGAAT CCCAGTGATA  369

WT-1 #1  TGCTCCTGCT GGTGGCCACA GCTTGGCATG GTCAGGGAAT CCCAGTGATA
WT-1 #2  TGCTCCTGCT GGTGGCCACA GCTTGGCATG GTCAGGGAAT CCCAGTGATA

Hu c-fms GAGCCCAGTG TCCCCGAGCT GGTCGTGAAG CCAGGAGCAA CGGTGACCTT  419

WT-1 #1  GAGCCCAGTG TCCCCGAGCT GGTCGTGAAG CCAGGAGCAA CGGTGACCTT
WT-1 #2  GAGCCCAGTG TCCCCGAGCT GGTCGTGAAG CCAGGAGCAA CGGTGACCTT

Hu c-fms GCGATGTGTG GGCAATGGCA GCGTGGAATG GGATGGCCCC GCATCACCTC  469

WT-1 #1  GCGATGTGTG GGCAATGGCA GCGTGGAATG GGATGGCCCC CCATCACCTC
WT-1 #2  GCGATGTGTG GGCAATGGCA GCGTGGAATG GGATGGCCCC CCATCACCTC

Hu c-fms ACTGGACCCT GTACTCTGAT GGCTCCAGCA GCATCCTCAG CACCAACAAC  519

WT-1 #1  ACTGGACCCT GTACTCTGAT GGCTCCAGCA GCATCCTCAG CACCAACAAC
WT-1 #2  ACTGGACCCT GTACTCTGAT GGCTCCAGCA GCATCCTCAG CACCAACAAT

Hu c-fms GCTACCTTCC AAAACACGGG GACCTATCGC TGCACTGAGC CTGGAGACCC  569

WT-1 #1  GCTACCTTCC AAAACACGGG GACCTATCGC TGCACTGAGC CTGGAGACCC
WT-1 #2  GCTACCTTCC AAAACACGGG GACCTATCGC TGCACTGAGC CTGGATACCC

Hu c-fms CCTGGGAGGC AGCGCCGCCA TCCACCTCTA TGTCAAAGAC CCTG-CCCGG  619

WT-1 #1  CCTGGGAGGC AGCGCCGCCA TCCATCTCTA TGTCAAAGAC CCTG-CCCGG
WT-1 #2  CCTGGGAGGC ATCGCCGCCA TCCACCTCTA TGTCAA-GAC CCTGGCCCGG

Hu c-fms CCCTGGA  625

WT-1 #1  CCCTGGT
WT-1 #2  CCCTGG-
```

```
Hu c-fms GTGCTGTTGA CCAATGGTCA TGTGGCCAAG ATTGGGGACT TCGGGCTGGC  2699

WT-1 #1  -TGCTGTTGA CCAATGGTCA TGTGGCCAAG ATTGGGGACT TCGGGCTGGC
WT-1 #2  GTGCTGTTGA CCAATGGTCA TGTGGCCAAG ATTGGGGACT TCGGGCTGGC

Hu c-fms TAGGGACATC ATGAATGACT CCAACTACAT TGTCAAGGGC AATGCCCGCC  2749

WT-1 #1  TAGGGACATC ATGAATGACT CCAACTACAT TGTCAAGGGC AATGCCCGCC
WT-1 #2  TAGGGACATC ATGAATGACT CCAACTACAT TGTCAAGGGC AATGCCCGCC

Hu c-fms TGCCTGTGAA GTGGATGGCC CCAGAGAGCA TCTTTGACTG TGTCTACACG  2799

WT-1 #1  TGCCTGTGAA GTGGATGGCC CCAGAGAGCA TCTTTGACTG TGTCTACACG
WT-1 #2  TGCCTGTGAA GTGGATGGCC CCAGAGAGCA TCTTTGACTG TGTCTACACG

Hu c-fms GTTCAGAGCG ACGTCTGGTC CTATGGCATC CTCCTCTGGG AGATCTTCTC  2849

WT-1 #1  GTTCAGAGCG ACGTCTGGTC CTATGGCATC CTCCTCTGGG AGATCTTCTC
WT-1 #2  GTTCAGAGCG ACGTCTGGTC CTATGGCATC CTCCTCTGGG AGATCTTCTC

Hu c-fms ACTTGGGCTG AATCCCTACC CTGGCATCCT GGTGAACAGC AAGTTCTATA  2899

WT-1 #1  ACTTGGGCTG AATCCCTACC CTGGCATCCT GGTGAACAGC AAGTTCTATA
WT-1 #2  ACTTGGGCTG AATCCCTACC CTGGCATCCT GGTGAACAGC AAGTTCTATA

Hu c-fms AACTGGTGAA GGATGGATAC CAAATGGCCC AGCCTGCATT TGCCCCAAAG  2949

WT-1 #1  AACTGGTGAA GGATGGATAC CAAATGGCCC AGCCTGCATT TGCCCCAAAG
WT-1 #2  AACTGGTGAA GGATGGATAC CAAATGGCCC AGCCTGCATT TGCCCCAAAG

Hu c-fms AATATATACA GCATCATGCA GGCCTGCTGG GCCTTGGAGC CCACCCACAG  2999

WT-1 #1  AATATATACA GCATCATGCA GGCCTGCTGG GCCTTGGAGC CCACCCACAG
WT-1 #2  AATATATACA GCATCATGCA GGCCTGCTGG GCCTTGGAGC CCACCCACAG

Hu c-fms ACCCACCTTC CAGCAGATCT GCTCCTTCCT TCAGGAGCAG GCCCAAGAGG  3049

WT-1 #1  ACCCACCTTC CAGCAGATCT GCTCCTTCCT TCAGGAGCAG GCCCAAGAGG
WT-1 #2  ACCCACCTTC CAGCAGATCT GCTCCTTCCT TCAGGAGCAG GCCCAAGAGG

Hu c-fms ACAGGAGAGA GCGGGACTAT ACCAATCTGC CGAGCAGCAG CAGAAGCGGT  3099

WT-1 #1  ACAGGAGAGA GCGGGACTAT ACCAATCTGC CGAGCAGCAG CAGAAGCGGT
WT-1 #2  ACAGGAGAGA GCGGGACTAT ACCAATCTGC CGAGCAGCAG CAGAAGCGGT

Hu c-fms GGCAGCGGCA GCAGCAGCAG TGAGCTGGAG GAGGAGAGCT CTAGTGAGCA  3149

WT-1 #1  GGCAGCGGCA GCAGCAGCAG TGAGCTGGAG GAGGAGAGCT CTAGTGAGCA
WT-1 #2  GGCAGCGGCA GCAGCAGCAG TGAGCTGGAG GAGGAGAGCT CTAGTGAGCA

Hu c-fms CCTGACCTGC TGCGAGCAAG GGGATATCGC CCAGCCCTTG CTGCAGCCCA  3199

WT-1 #1  CCTGACCTGC TGCGAGCAAG GGGATATCGC CCAGCCCTTG CTGCAGCCCA
WT-1 #2  CCTGACCTGC TGCGAGCAAG GGGATATCGC CCAGCCCTTG CTGCAGCCCA

Hu c-fms ACAACTATCA GTTCTGCTGA GGAGTTGACG 3229

WT-1 #1  ACAACTATCA GTTCTGCTGA GGAGT-----
WT-1 #2  ACAACTATCA GTTCTGCTGA GGAGT-----
```

Multiple sequence alignment of wild-type myc/his tagged human c-fms in pcDNA3.1^{+}.

```
SR-1          ---------- ---------A ACGGCCGCCA GTG-TGCTGG AATTCGGCTT  30
T7            ----CGGATC CAG-GGCCCA ACGGCCGCCA GTGANGCTGG AATTCGGCTT  45
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          ---------- ---------- ---------- ---------- ----------
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ----------
SF-1          ---------- ---------- ---------- ---------- ----------
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     CCTGGGCACC TTGCAGCCCT GCACCTGCC- -TGCCACTTC CCCACCGAGG  298
Consensus     CCTGSGSAYC YWGCRGCCCW RCRSCYGCCA GTGMNRCTKS MMYWCSGMKK  300

SR-1          CCATGGGCCC AGGAGTTCTG CTGCTCCTGC TGGTGGCCAC AGCTTGGCAT  80
T7            CCATGGGCCC AGGAGTTCTG CTGCTCCTGC TGGTGGCCAC AGCTTGGCAT  95
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          ---------- ---------- ---------- ---------- ----------
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ----------
SF-1          ---------- ---------- ---------- ---------- ----------
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     CCATGGGCCC AGGAGTTCTG CTGCTCCTGC TGGTGGCCAC AGCTTGGCAT  348
Consensus     CCATGGGCCC AGGAGTTCTG CTGCTCCTGC TGGTGGCCAC AGCTTGGCAT  350

SR-1          GGTCAGGGAA TCCCAGTGAT AGAGCCCAGT GTCCCCGAGC TGGTCGTGAA  130
T7            GGTCAGGGAA TCCCAGTGAT AGAGCCCAGT GTCCCCGAGC TGGTCGTGAA  145
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          ---------- ---------- ---------- ---------- ----------
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ----------
SF-1          ---------- ---------- ---------- ---------- ----------
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GGTCAGGGAA TCCCAGTGAT AGAGCCCAGT GTCCCCGAGC TGGTCGTGAA  398
Consensus     GGTCAGGGAA TCCCAGTGAT AGAGCCCAGT GTCCCCGAGC TGGTCGTGAA  400

SR-1          GCCAGGAGCA ACGGTGACCT TGCGATGTGT GGGCAATGGC AGCGTGGAAT  180
T7            GCCAGGAGCA ACGGTGACCT TGCGATGTGT GGGCAATGGC AGCGTGGAAT  195
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          ---------- ---------- ---------- ---------- ----------
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ----------
SF-1          ---------- ---------- ---------- ---------- ----------
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GCCAGGAGCA ACGGTGACCT TGCGATGTGT GGGCAATGGC AGCGTGGAAT  448
Consensus     GCCAGGAGCA ACGGTGACCT TGCGATGTGT GGGCAATGGC AGCGTGGAAT  450

SR-1          GGGATGGCCC CCCATCACCT CACTGGACCC TGTACTCTGA TGGCTCCAGC  230
T7            GGGATGGCCC CCCATCACCT CACTGGACCC TGTACTCTGA TGGCTCCAGC  245
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          ---------- ---------- ---------- ---------- ----------
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ----------
SF-1          ---------- ---------- ---------- ---------- ----------
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GGGATGGCCC CGCATCACCT CACTGGACCC TGTACTCTGA TGGCTCCAGC  498
```

```
Consensus       GGGATGGCCC CSCATCACCT CACTGGACCC TGTACTCTGA TGGCTCCAGC   500


SR-1            AGCATCCTCA GCACCAACAA CGCTACCTTC CAAAACACGG GGACCTATCG   280
T7              AGCATCCTCA GCACCAACAA CGCTACCTTC CAAAACACGG GGACCTATCG   295
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------
SF-1            ---------- ---------- ---------- ---------- ----------
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       AGCATCCTCA GCACCAACAA CGCTACCTTC CAAAACACGG GGACCTATCG   548
Consensus       AGCATCCTCA GCACCAACAA CGCTACCTTC CAAAACACGG GGACCTATCG   550


SR-1            CTGCACTGAG CCTGGAGACC CCCTGGGAGG CAGCGCCGCC ATCCACCTCT   330
T7              CTGCACTGAG CCTGGAGACC CCCTGGGAGG CAGCGCCGCC ATCCACCTCT   345
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------
SF-1            ---------- ---------- ---------- ---------- ----------
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CTGCACTGAG CCTGGAGACC CCCTGGGAGG CAGCGCCGCC ATCCACCTCT   598
Consensus       CTGCACTGAG CCTGGAGACC CCCTGGGAGG CAGCGCCGCC ATCCACCTCT   600


SR-1            ATGT--AAGA CCCTGCCCGG ---------- ---------- ----------   348
T7              ATGTCAAAGA CCCTGCCCGG CCCTGGAACG TGCTAGCACA GGAGGTGGTC   395
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------
SF-1            ---------- ---------- ---------- ---------- ----------
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       ATGTCAAAGA CCCTGCCCGG CCCTGGAACG TGCTAGCACA GGAGGTGGTC   648
Consensus       ATGTCAAAGA CCCTGCCCGG CCCTGGAACG TGCTAGCACA GGAGGTGGTC   650


SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              GTGTTCGAGG ACCAGGACGC ACTACTGCCC TGTCTGCTCA CAGACCCGGT   445
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------
SF-1            ---------- ---------- ---------- ---------- ----------
BGH             ---------- ---------- ---------- ---------- ----------
Sr-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       GTGTTCGAGG ACCAGGACGC ACTACTGCCC TGTCTGCTCA CAGACCCGGT   698
Consensus       GTGTTCGAGG ACCAGGACGC ACTACTGCCC TGTCTGCTCA CAGACCCGGT   700


SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              GCTGGAAGCA WGCGTCTCGC TGGTGCGTGT GCGTGGCCGG CCC-TCATGC   494
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------
SF-1            ---------- ---------- ---------- ---------- ----------
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       GCTGGAAGCA GGCGTCTCGC TGGTGCGTGT GCGTGGCCGG CCCCTCATGC   748
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Consensus       GCTGGAAGCA NGCGTCTCGC TGGTGCGTGT GCGTGGCCGG CCCCTCATGC  750

SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              GCCACACCAA CTACTCCTTC TCGCCCTGGC ATGGCTTCAC CATCCACAGG  544
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- --------GC ATGGTTTCAC CATCCACAGG   22
SF-1            ---------- --ACTAC-CC TCGCCCTGGC AAAMMTTCAC CATCCACAGG   37
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       GCCACACCAA CTACTCCTTC TCGCCCTGGC ATGGCTTCAC CATCCACAGG  798
Consensus       GCCACACCAA CTACTMCTYC TCGCCCTGGC AMGMMTTCAC CATCCACAGG  800

SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              GCCAAGTTCA TTCAGAGCCA GGACTATCAA TGCAGTGCCC TGATGGGTGG  594
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            GCCAAGTTCA TT-AGAGCCA GGANTATCAN TGCAGTGCCT TGATGG-TTG   70
SF-1            GCCAAGTTCA TTCAGAGCCA GGACTATCAA TGCAGTGCCC TGATGGGTGG   87
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       GCCAAGTTCA TTCAGAGCCA GGACTATCAA TGCAGTGCCC TGATGGGTGG  848
Consensus       GCCAAGTTCA TTCAGAGCCA GGANTATCAN TGCAGTGCCY TGATGGGTKG  850

SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              CAGGAAGGTG ATGT-CATCA GCATCCGGCT GAAAGTG-AA AAAGTCATCC  642
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            CAGGAAGNTG ATG-NCATCA GCATCCGGCT G-AAGTGCAG AAAGTCATCC  118
SF-1            CAGGAAGGTG ATGTCCATCA GCATCCGGCT GAAAGTGCAG AAAGTCATCC  137
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CAGGAAGGTG ATGTCCATCA GCATCCGGCT GAAAGTGCAG AAAGTCATCC  898
Consensus       CAGGAAGNTG ATGTNCATCA GCATCCGGCT GAAAGTGCAR AAAGTCATCC  900

SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              AAGGG-CCCC AGCCTTGACA CTGGTGCCTG CA-AGCTGGT GCGGNTTC-A  689
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            CAGGGCCCCC AGCCTTGACA ATTGTGCNTG CAGAGTTGGT GCGGATTCGA  168
SF-1            CAGGGCCCCC AGCCTTGACA CTGGTGCCTG CAGAGCTGGT GCGGATTCGA  187
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CAGGGCCCCC AGCCTTGACA CTGGTGCCTG CAGAGCTGGT GCGGATTCGA  948
Consensus       MAGGGCCCCC AGCCTTGACA MTKGTGCNTG CAGAGYTGGT GCGGNTTCGA  950

SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              NGGGAAGCTG CC-AAATNGT GTG-TCAACC AACAACGTTG ATGTTAACTT  737
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            GGGGAGGTTG CCCAGATCGT GTGNTCAGCC AGCAGCGTTG ATGTTAACTT  218
SF-1            GGGGAGGCTG CCCAGATCGT GTGCTCAGCC AGCAGCGTTG ATGTTAACTT  237
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
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C-fms Tag       GGGGAGGCTG CCCAGATCGT GTGCTCAGCC AGCAGCGTTG ATGTTAACTT   998
Consensus       NGGGARGYTG CCCARATMGT GTGMTCARCC ARCARCGTTG ATGTTAACTT   1000



SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              TGATGTCT-C CT-CAACA-A ACAACACTAA GCTCGCAAIN CCTCAA-AAT   783
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            TGATGTCTTC CTCCAACACA ACAACACTAA GCTCGCAATC CCTCAACAAT   268
SF-1            TGATGTCTTC CTCCAACACA ACAACACTAA GCTCGCAATC CCTCAACAAT   287
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       TGATGTCTTC CTCCAACACA ACAACACTAA GCTCGCAATC CCTCAACAAT   1048
Consensus       TGATGTCTTC CTCCAACACA ACAACACTAA GCTCGCAAIN CCTCAACAAT   1050


SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              CTGACTTTCA TA-TAANCGN TNC--AAAAA TCCTGACCCT CA-CCTC-AT   828
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            CTGACTTTCA TAATAACCGT TACCAAAAAG TCCTGACCCT CAACCTCGAT   318
SF-1            CTGACTTTCA TAATAACCGT TACCAAAAAG TCCTGACCCT CAACCTCGAT   337
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CTGACTTTCA TAATAACCGT TACCAAAAAG TCCTGACCCT CAACCTCGAT   1098
Consensus       CTGACTTTCA TAATAANCGN TNCCAAAAAR TCCTGACCCT CAACCTCGAT   1100


SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              NAAGT----- ---------- ---------- ---------- ----------   833
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---------- ---------- ---------- ---------- ----------
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            CAAGTAGATT TCCAACATGC CGGCAACTAC TCCTGCGTGG CCAGCAACGT   368
SF-1            CAAGTAGATT TCCAACATGC CGGCAACTAC TCCTGCGTGG CCAGCAACGT   387
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CAAGTAGATT TCCAACATGC CGGCAACTAC TCCTGCGTGG CCAGCAACGT   1148
Consensus       NAAGTAGATT TCCAACATGC CGGCAACTAC TCCTGCGTGG CCAGCAACGT   1150


SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              ---------- ---------- ---------- ---------- ----------   833
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            ---AGGNAAG CANTCCACCT C-ATNTTNTT CC-GGTTGTA GNNANT--CC   43
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            GCAGGGCAAG CACTCCACCT CCATGTTCTT CCGGGTGGTA GAGAGTGCCT   418
SF-1            GCAGGGCAAG CACTCCACCT CCATGTTCTT CCGGGTGGTA GAGAGTGCCT   437
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       GCAGGGCAAG CACTCCACCT CCATGTTCTT CCGGGTGGTA GAGAGTGCCT   1198
Consensus       GCARGGNAAG CANTCCACCT CCATNTTNTT CCGGGTKGTA GNNANTGCCY   1200


SR-1            ---------- ---------- ---------- ---------- ----------   348
T7              ---------- ---------- ---------- ---------- ----------   833
SR-5            ---------- ---------- ---------- ---------- ----------
SF-2            ---------- ---------- ---------- ---------- ----------
SR-4            AATTGAACTT GAGNTTTG-G CAGANCCTCA TCCAGGAG-T GACCNTGGGG   91
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ACTTGAACTT GAGCTCTGAG CAGAACCTCA TCCAGGAGGT GACCGTGGGG   468
SF-1            ACTTGAACTT GAGCTCTGAG CAGAACCTCA TCCAGGAGGT GACCGTGGGG   487
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
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SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     ACTTGAACTT GAGCTCTGAG CAGAACCTCA TCCAGGAGGT GACCGTGGGG  1248
Consensus     AMTTGAACTT GAGMTYTGAG CAGAMCCTCA TCCAGGAGGT GACCMTGGGG  1250


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          --AGGGMTCA ACCTCAAAGT CATG-TGGAG GCMTACCCAG GCMTG-MAAG  137
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          GAGGGGCTCA ACCTCAAAGT CATGGTGGAG GC-TACCCAG GCCTGCAAGG  517
SF-1          GAAGGGCTCA ACCTCAAAGT CATGGTGGAG GC-TACCCAA GCCTGCAAGG  536
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GAGGGGCTCA ACCTCAAAGT CATGGTGGAG GCCTACCCAG GCCTGCAAGG 1298
Consensus     GARGGGMTCA ACCTCAAAGT CATGGTGGAG GCMTACCCAR GCMTGCMAMG 1300


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          TTTTAACTGG ACCTACTT-G GACCCTTTTT TGACCACCAG CMTGAGCCCA  186
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          TTTTAACTGG ACCTACCTGG GACCCTTTTC TGACCACCAG CCTGAGCCCA  567
SF-1          TTTTAACTGG ACCTACCTGG GACCCTTTTC TGACCAACAG CCTGAGCC-A  585
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     TTTTAACTGG ACCTACCTGG GACCCTTTTC TGACCACCAG CCTGAGCCCA 1348
Consensus     TTTTAACTGG ACCTACYTGG GACCCTTTTY TGACCAMCAG CMTGAGCCCA 1350


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          NGCTTGCTAA TGTTACCACC AAGGACACAT ACAGGCACAC CTTCACCMTC  236
SF-3          ********** ********** ---------- ---------- ----------
SR-3          AGCTTGCTAA TGCTACCACC AAGGACACAT ACAGGCACAC CTTCACCCTC  617
SF-1          AGCMTGCTAA TGCTAMCACC AAGGMCACAT ACAMGMACAC CT-CAACCTC  634
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     AGCTTGCTAA TGCTACCACC AAGGACACAT ACAGGCACAC CTTCACCCTC 1398
Consensus     NGCMTGCTAA TGYTAMCACC AAGGMCACAT ACAMGMACAC CTTCAMCMTC 1400


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          TCTTTGCCCC GCTTGAAGCC CTCTGAGGCT GGCCGCTACT CCTTCCTGGC  286
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          TCTCTGCCCC GCCTGAAGCC CTCTGAGGCT GGCCGCTACT CCTTCCTGGC  667
SF-1          TCTCTGCCCC GCCTGAA-MC CTCTGAMGGT GGCCGCTAMT CCTTCCTGGG  683
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     TCTCTGCCCC GCCTGAAGCC CTCTGAGGCT GGCCGCTACT CCTTCCTGGC 1448
Consensus     TCTTTGCCCC GCTTGAAGMC CTCTGAMGST GGCCGCTAMT CCTTCCTGGS 1450


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------
SF-2          ---------- ---------- ---------- ---------- ----------
SR-4          CAGAAACCCA GGAGGMTGGA GAGCTCTGAC GTTTGAGCTC ACCCTTCGAT  336
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          CAGAAACCCA GGAGGCTGGA GAGCTCTGAC GTTTGAGCTC ACCCTTCGAT  717
SF-1          CAAAAANCCA AGAGGMTGGM -AACTCTGAC -TTTGAGCCC AACCTT-GA-  729
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
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SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     CAGAAACCCA GGAGGCTGGA GAGCTCTGAC GTTTGAGCTC ACCCTTCGAT 1498
Consensus     CARAAANCCA RGAGGNTGGN GARCTCTGAC GTTTGAGCTC ANCCTTCGAT 1500


SR-1          ---------- ---------- ---------- ---------- ---------- 348
T7            ---------- ---------- ---------- ---------- ---------- 833
SR-5          ---------- ---------- ---------- ---------- ----TGCA-C 5
SF-2          ---------- ---------- ---------- ------ACGG CTCTGGCN-C 13
SR-4          ACCCCCCAGA GGTAAGCGTC ATATGGACAT TCATCAACGG CTCTGGCACC 386
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ACCCCCCAGA GGTAAGCGTC ATTTNGACAT TCATCAA--- ---------- 754
SF-1          NACCCCCAAA GGGAA-CGTC ANAT-GGCAA TCA-CAACGG C--------- 767
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     ACCCCCCAGA GGTAAGCGTC ATATGGACAT TCATCAACGG CTCTGGCACC 1548
Consensus     NNCCCCCARA GGNAAGCGTC ANNTNGRCAN TCATCAACGG CTCTNGCNCC 1550


SR-1          ---------- ---------- ---------- ---------- ---------- 348
T7            ---------- ---------- ---------- ---------- ---------- 833
SR-5          CCTTTGTGTG -TGCCTTT-G GTACCCCCAG -CCNAAGTGA CATGGTT-NN 51
SF-2          CTTTTGTGTG CTAAATCTGG GTACCCCCAG CCCAACGTGA CATGGCTGCA 63
SR-4          CTTTTGTGTG CTGCCTCTGG GTACCCCCAG CCCAACGTGA CATGGCTGCA 436
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ---------- 754
SF-1          ---------- ------CTGG G-------AN CC--------- ---------- 776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     CTTTTGTGTG CTGCCTCTGG GTACCCCCAG CCCAACGTGA CATGGCTGCA 1598
Consensus     CTTTTGTGTG CTNNCTYTGG GTACCCCCAN CCCNAMGTGA CATGGYTGNN 1600


SR-1          ---------- ---------- ---------- ---------- ---------- 348
T7            ---------- ---------- ---------- ---------- ---------- 833
SR-5          GTGCAGT-GC CACANTGATA G-TNTGATGA GGCCCAAGTG TTGCAGGTTT 99
SF-2          GTGCAGTGGC CACACTGATA GGTGTGATGA GGCCCAAGTG CTGCAGGTCT 113
SR-4          GTGCAGTGGC CACACTGATA GGTGTGATGA GGCCCAAGTG CTGCAGGTCT 486
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ---------- 754
SF-1          ---------- ---------- ---------- ---------- ---------- 776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GTGCAGTGGC CACACTGATA GGTGTGATGA GGCCCAAGTG CTGCAGGTCT 1648
Consensus     GTGCAGTGGC CACANTGATA GGTNTGATGA GGCCCAAGTG YTGCAGGTYT 1650


SR-1          ---------- ---------- ---------- ---------- ---------- 348
T7            ---------- ---------- ---------- ---------- ---------- 833
SR-5          -GGATGACCC ATACCNTGAG -TCNTGAGCC AGGAGCCNTT CCACAAGGTG 147
SF-2          GGGATGACCC ATACCCTGAG GTCCTGAGCC AGGAGCCCTT CCAGAAGGTG 163
SR-4          GGGATGACCC ATACCCTGAG GTCCTGAGCC AGGAGCCCTT CCACAAGGTG 536
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ---------- 754
SF-1          ---------- ---------- ---------- ---------- ---------- 776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GGGATGACCC ATACCCTGAG GTCCTGAGCC AGGAGCCCTT CCACAAGGTG 1698
Consensus     GGGATGACCC ATACCNTGAG GTCNTGAGCC AGGAGCCNTT CCASAAGGTG 1700


SR-1          ---------- ---------- ---------- ---------- ---------- 348
T7            ---------- ---------- ---------- ---------- ---------- 833
SR-5          ACGGTGCAGA NCTTGCTGAT TGTTGAGNCC TTAGAGCACA NCCAAACCTA 197
SF-2          ACGGTGCAGA GCCTGCTGAC TGTTGAGACC TTAGAGCACA ACCAAACCTA 213
SR-4          ACGGTGCAGA GCCTGCTGAC TGTTGAGACC TTAGAGCACA ACCAAACCTA 586
SF-3          ---------- ---------- ---------- ---------- ----------
SR-3          ---------- ---------- ---------- ---------- ---------- 754
SF-1          ---------- ---------- ---------- ---------- ---------- 776
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BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       ACGGTGCAGA GCCTGCTGAC TGTTGAGACC TTAGAGCACA ACCAAACCTA  1748
Consensus       ACGGTGCAGA NCTTGCTGAY TGTTGAGNCC TTAGAGCACA NCCAAACCTA  1750


SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              ---------- ---------- ---------- ---------- ----------  833
SR-5            CGAGTGCAGG GCCCACAACA GCGTGGGGAG TGGCTCTTGG CCCTTCATAC  247
SF-2            CGAGTGCAGG GCCCACAACA GCGTGGGGAG TGGCTCCTGG GCCTTCATAC  263
SR-4            CGAGTGCAGG GCCCACAACA GCGTGGGGAG TGGCTCCTGG GCCTTCATAC  636
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------  754
SF-1            ---------- ---------- ---------- ---------- ----------  776
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CGAGTGCAGG GCCCACAACA GCGTGGGGAG TGGCTCCTGG GCCTTCATAC  1798
Consensus       CGAGTGCAGG GCCCACAACA GCGTGGGGAG TGGCTCYTGG SCCTTCATAC  1800


SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              ---------- ---------- ---------- ---------- ----------  833
SR-5            CCATCTCTGC AGGAGCCCAC ACGCATCCCC CGGATGAGTT CCTCTTCACA  297
SF-2            CCATCTCTGC AGGAGCCCAC ACGCATCCCC CGGATGAGTT CCTCTTCACA  313
SR-4            CCATCTCTGC AGGAGCCCAC ACGCATCCCC CGGATGAGTT CCTCTTCACA  686
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------  754
SF-1            ---------- ---------- ---------- ---------- ----------  776
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CCATCTCTGC AGGAGCCCAC ACGCATCCCC CGGATGAGTT CCTCTTCACA  1848
Consensus       CCATCTCTGC AGGAGCCCAC ACGCATCCCC CGGATGAGTT CCTCTTCACA  1850


SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              ---------- ---------- ---------- ---------- ----------  833
SR-5            CCAGTGGTGG TCGCCTGCAT GTCCATCATG GCCTTGCTGC TGCTGCTGCT  347
SF-2            CCAGTGGTGG TCGCCTGCAT GTCCATCATG GCCTTGCTGC TGCTGCTGCT  363
SR-4            CCAGTGGTGG TCGCCTGCAT GTCCATCATG GCCTTGCTGC TGCTGCTGCT  736
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------  754
SF-1            ---------- ---------- ---------- ---------- ----------  776
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CCAGTGGTGG TCGCCTGCAT GTCCATCATG GCCTTGCTGC TGCTGCTGCT  1898
Consensus       CCAGTGGTGG TCGCCTGCAT GTCCATCATG GCCTTGCTGC TGCTGCTGCT  1900


SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              ---------- ---------- ---------- ---------- ----------  833
SR-5            CCTGCTGCTA TTGTACAAGT ATAAGCAGAA GCCCAAGTAC CAGGTCCGCT  397
SF-2            CCTGCTGCTA TTGTACAAGT ATAAGCAGAA GCCCAAGTAC CAGGTCCGCT  413
SR-4            CCTGCTGCTA TTGTACAAGT ATAAGCAGAA GCCCAAGTAC CAGG------  780
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------  754
SF-1            ---------- ---------- ---------- ---------- ----------  776
BGH             ---------- ---------- ---------- ---------- ----------
SF-5            ---------- ---------- ---------- ---------- ----------
SF-4            ---------- ---------- ---------- ---------- ----------
SR-6            ---------- ---------- ---------- ---------- ----------
C-fms Tag       CCTGCTGCTA TTGTACAAGT ATAAGCAGAA GCCCAAGTAC CAGGTCCGCT  1948
Consensus       CCTGCTGCTA TTGTACAAGT ATAAGCAGAA GCCCAAGTAC CAGGTCCGCT  1950


SR-1            ---------- ---------- ---------- ---------- ----------  348
T7              ---------- ---------- ---------- ---------- ----------  833
SR-5            GGAAGATCAT CGAGAGCTAT GAGGGCAACA GTTATACTTT CATCGACCCC  447
SF-2            GGAAGATCAT CGAGAGCTAT GAGGGCAACA GTTATACTTT CATCGACCCC  463
SR-4            ---------- ---------- ---------- ---------- ----------  780
SF-3            ---------- ---------- ---------- ---------- ----------
SR-3            ---------- ---------- ---------- ---------- ----------  754
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SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     GGAAGATCAT CGAGAGCTAT GAGGGCAACA GTTATACTTT CATCGACCCC 1998
Consensus     GGAAGATCAT CGAGAGCTAT GAGGGCAACA GTTATACTTT CATCGACCCC 2000


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ACGCAGCTGC CTTACAACGA GAAGTGGGAG TTCCCCCGGA ACAACCTGCA  497
SF-2          ACGCAGCTGC CTTACAACGA GAAGTGGGAG TTCCCCCGGA ACAACCTGCA  513
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- -------GGA ACAACCTGCA   13
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ---------- ---------- ----------
C-fms Tag     ACGCAGCTGC CTTACAACGA GAAGTGGGAG TTCCCCCGGA ACAACCTGCA 2048
Consensus     ACGCAGCTGC CTTACAACGA GAAGTGGGAG TTCCCCCGGA ACAACCTGCA 2050


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          GTTTGGTAAG ACCCTCGGAG CTGGAGCC-- --TTTGGGAA GGTGGTGGAG  543
SF-2          GTTTGGTAAG ACCCTCGGAG CTGGAGCC-- --TTTGGGAA GGTGGTGGAG  559
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          GTTTGGTAAG ACCCTCGGAG CTGGAGCC-- --TTTGGGAA GGTGGTGGAG   59
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ------CCTT TGTTTGGCAA GG--------   16
C-fms Tag     GTTTGGTAAG ACCCTCGGAG CTGGAGCCTT TG----GGAA GGTGGTGGAG 2094
Consensus     GTTTGGTAAG ACCCTCGGAG CTGGAGCCTT TGTTTGGSAA GGTGGTGGAG 2100


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          GCCACGGCCT TTGGTCTGGG CAAGGAGGAT GCTGTCCTGA AGGTGGCTGT  593
SF-2          GCCACGGMCT TTGGTCTGGG CAAGGAGGAT GCTGTCCTGA AGGTGGCTGT  609
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          GCCACGGCCT TTGGTCTGGG CAAGGAGGAT GCTGTCCTGA AGGTGGCTGT  109
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          ---------- ---------- ------GGAT GTT-TCCT-A AGGTGGMT-T   37
C-fms Tag     GCCACGGCCT TTGGTCTGGG CAAGGAGGAT GCTGTCCTGA AGGTGGCTGT 2144
Consensus     GCCACGGMCT TTGGTCTGGG CAAGGAGGAT GYTGTCCTGA AGGTGGMTGT 2150


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          GAAGATGCTG AAGTCCACGG CCCATGCTGA TGACAAGCAG CCCCTCATGT  643
SF-2          GAAGATGCTG AAGTCCACGG CC-ATGCTGA TGAMAAGGAG GCC-TCATGT  657
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          GAAGATGCTG AAGTCCACGG CCCATGCTGA TGAGAAGGAG GCCCTCATGT  159
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          G-AGGTTCTA AAG-CCA-GG GCCATGTT-A TG-GGAGGA- GCCCNTATNN   81
C-fms Tag     GAAGATGCTG AAGTCCACGG CCCATGCTGA TGAGAAGGAG GCCCTCATGT 2194
Consensus     GAAGRTKCTR AAGTCCACGG SCCATGYTGA TGANRAGGAG GCCCNYATNN 2200


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          CCGAGCTGAA GATCATGAGC CACCTGGGCC AGCACGAGAA CATCGTCAAC  693
SF-2          CC-AGCTGAA NATNATGAGC CANCTGGGC- AACACGAGAA CATC-TCAAA  704
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CCGAGCTGAA GATCATGAGC CACCTGGGCC AGCACGAGAA CATCGTCAAC  209
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SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          CCGAGTT-AA NATC-TTNCC CACTTGGCCC AGCA-NAGAA CAT-GTCACC  127
C-fms Tag     CCGAGCTGAA GATCATGAGC CACCTGGGCC AGCACGAGAA CATCGTCAAC  2244
Consensus     CCGAGTTGAA NATNATTNSC CANYTGGSCC ARCACNAGAA CATCGTCANN  2250


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          CTTCTGGGAG CCTGTACCCA TGGAGGCCCT GTACTGGTCA TCACGGAGTA  743
SF-2          CTTCTGGGAA CCTGTNCCCA TGGAGGGCCT GTACTGGNCA TCACGG-GTA  753
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CTTCTGGGAG CCTGTACCCA TGGAGGCCCT GTACTGGTCA TCACGGAGTA  259
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          CTTTTGG--A CCTGTA-CCA TTGAGGCCNT GNATTGGTCA TCACGGAGTA  174
C-fms Tag     CTTCTGGGAG CCTGTACCCA TGGAGGCCCT GTACTGGTCA TCACGGAGTA  2294
Consensus     CTTYTGGGAR CCTGTNCCCA TRGAGGSCNT GNAYTGGNCA TCACGGAGTA  2300


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          CTGTTGCTAT GGCGACCTGC TCAACTTTCT GCGAAGGAAG GCTGAGGCCA  793
SF-2          C-GTTGC-AT GG-GAACTGC -CAACTTTCT GCAAAGGAAG G-TGAGG-CA  797
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CTGTTGCTAT GGCGACCTGC TCAACTTTCT GCGAAGGAAG GCTGAGGCCA  309
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          CTGTTGCTAT GGCGACNTG- TCAACTTTTT GCGAAGGAAG GCTGAGGCCA  223
C-fms Tag     CTGTTGCTAT GGCGACCTGC TCAACTTTCT GCGAAGGAAG GCTGAGGCCA  2344
Consensus     CTGTTGCTAT GGCGANNTGC TCAACTTTYT GCRAAGGAAG GCTGAGGCCA  2350


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          TGCTGGGACC CAGCCTGAGC CCCG------ ---------- ----------  817
SF-2          T-CTGGGA-C CAACCTAANC CCGG------ ---------- ----------  819
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          TGCTGGGACC CAGCCTGAGC CCCGGCCAGG ACCCCGAGGG AGGCGTCGAC  359
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          TGCTGG-ACC CAGCTTGAGC CCCGGCCAGG ACCCCGAGGG AGGCGTCGAC  272
C-fms Tag     TGCTGGGACC CAGCCTGAGC CCCGGCCAGG ACCCCGAGGG AGGCGTCGAC  2394
Consensus     TGCTGGGACC CARCYTRAGC CCSGGCCAGG ACCCCGAGGG AGGCGTCGAC  2400


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  819
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          TATAAGAACA TCCACCTCGA GAAGAAATAT GTCCGCAGGG ACAGTGGCTT  409
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- ---------- ---------- ----------
SR-6          TATAAGAACA TCCACCTCGA GAAGAAATAT GTCCGCAGGG ACAGTGGCTT  322
C-fms Tag     TATAAGAACA TCCACCTCGA GAAGAAATAT GTCCGCAGGG ACAGTGGCTT  2444
Consensus     TATAAGAACA TCCACCTCGA GAAGAAATAT GTCCGCAGGG ACAGTGGCTT  2450


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  819
SR-4          ---------- ---------- ---------- ---------- ----------  780
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SF-3          CTCCAGCCAN GGTGTGGACA CCTATGTGGA GATGAGGCCT GTCTCCACTT  459
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ---------- ---------- --------GA GATGAGGCCT GNAACCACTT   22
SR-6          CTCCAGCCAG GGTGTGGACA CCTATGTGGA GATGAGGCCT GTCTCCACTT  372
C-fms Tag     CTCCAGCCAG GGTGTGGACA CCTATGTGGA GATGAGGCCT GTCTCCACTT 2494
Consensus     CTCCAGCCAN GGTGTGGACA CCTATGTGGA GATGAGGCCT GNNNCCACTT 2500


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---CAAGACC ----CNAG-- ----------  830
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CTTCAAATGA CTCCTTCTCT GAGCAAGACC TGGACAAGGA GGATGGACGG  509
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ---------- ---------- ---------- ---------- ----------
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          CTTCAAATGA CTCCTTCTCT GAGCAAGACC TGGACAAGGA GGATGGACGG   72
SR-6          CTTCAAATGA CTCCTTCTCT GAGCAAGACC TGGACAAGGA GGATGGACGG  422
C-fms Tag     CTTCAAATGA CTCCTTCTCT GAGCAAGACC TGGACAAGGA GGATGGACGG 2544
Consensus     CTTCAAATGA CTCCTTCTCT GAGCAAGACC TGGACNAGGA GGATGGACGG 2550


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          -----GGA-- ---GGG---- ---TCACT-- ---A--TAA- ------AA--  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CCCCTGGAGC TCCGGGACCT GCTTCACTTC TCCAGCCAAG TAGCCCAAGG  559
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ------GAGC TCCGGG-CCT G-TTCAATTN TCCAG-CAAG TAGCCCAGG-   40
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          CCCCTGGAGC TCCGGGACCT GCTTCACTTC TCCAGCCAAG TAGCCCAGGG  122
SR-6          CCCCTGGAGC TCCGGGACCT GCTTCACTTC TCCAGCCAAG TAGCCCAGGG  472
C-fms Tag     CCCCTGGAGC TCCGGGACCT GCTTCACTTC TCCAGCCAAG TAGCCCAGGG 2594
Consensus     CCCCTGGAGC TCCGGGACCT GCTTCANTTN TCCAGCYAAG TAGCCCARGG 2600


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CATGGNCTTC CTCGCTTCCA AGAATTGCAT CCANCGGGAC GTGG-AACCC  608
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           CAT-GCCTTC CTNGTTTCCA GGANTTGCAT CCA-CGGGAA GTGGCAGCGC   88
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          CATGGCCTTC CTCGCTTCCA AGAATTGCAT CCACCGGGAC GTGGCAGCGC  172
SR-6          CATGGCCTTC CTCGCTTCCA AGAATTGCAT CCACCGGGAC GTGGCAGCGC  522
C-fms Tag     CATGGCCTTC CTCGCTTCCA AGAATTGCAT CCACCGGGAC GTGGCAGCGC 2644
Consensus     CATGGNCTTC CTNGYTTCCA RGANTTGCAT CCANCGGGAM GTGGCARCSC 2650


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          GTAACGTGCT GTTGAC-AAT GGTCATGTGG -CAAGANTGG GGACTTCGGG  656
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           GTAA-GTGNT GTTGACCANT GGTCATGTGC CCAGGATTGG GGACTTCGGG  137
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          GTAACGTGCT GTTGACCAAT GGTCATGTGG CCAAGATTGG GGACTTCGGG  222
SR-6          GTAACGTGCT GTTGACCAAT GGTCATGTGG CCAAGATTGG GGACTTCGGG  572
C-fms Tag     GTAACGTGCT GTTGACCAAT GGTCATGTGG CCAAGATTGG GGACTTCGGG 2694
Consensus     GTAACGTGNT GTTGACCANT GGTCATGTGS CCARGANTGG GGACTTCGGG 2700


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
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SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CTGGT-AAGG A-ATCATGAA TGACTCCAAC TACATT-TCA AGGGAAAT-N  702
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           TTGGTT-GGG ACATCATGAA TGACTCCAA- TACATTGTCA AGGGCAATGC  185
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          CTGGCTAGGG ACATCATGAA TGACTCCAAC TACATTGTCA AGGGCAATGC  272
SR-6          CTGGCTAGGG ACATCATGAA TGACTCCAAC TACATTGTCA AGGGCAATGC  622
C-fms Tag     CTGGCTAGGG ACATCATGAA TGACTCCAAC TACATTGTCA AGGGCAATGC 2744
Consensus     YTGGYTARGG ACATCATGAA TGACTCCAAC TACATTGTCA AGGGMAATGN 2750


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          CCGCCTGCCT GTMAA----- ---------- ---------- ----------  717
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           CCGCCTGCCT GTGAAGTGGA TGGCCCCAGA GAGCATCTTT GACTGTGTCT  235
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          CCGCCTGCCT GTGAAGTGGA TGGCCCCAGA GAGCATCTTT GACTGTGTCT  322
SR-6          CCGCCTGCCT GTGAAGTGGA TGGCCCCAGA GAGCATCTTT GACTGTGTCT  672
C-fms Tag     CCGCCTGCCT GTGAAGTGGA TGGCCCCAGA GAGCATCTTT GACTGTGTCT 2794
Consensus     CCGCCTGCCT GTGAAGTGGA TGGCCCCAGA GAGCATCTTT GACTGTGTCT 2800


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ------TTG- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           ACACGGTTCA GAGCGACGTC TGGTCCTATG GCATCCTCCT CTGGGAGATC  285
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          ACACGGTTCA GAGCGACGTC TGGTCCTATG GCATCCTCCT CTGGGAGATC  372
SR-6          ACACGGTTCA GAGCGACGTC TGGTCCTATG GCATCCTCCT CTGGGAGATC  722
C-fms Tag     ACACGGTTCA GAGCGACGTC TGGTCCTATG GCATCCTCCT CTGGGAGATC 2844
Consensus     ACACGGTTSA GAGCGACGTC TGGTCCTATG GCATCCTCCT CTGGGAGATC 2850


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           TTCTCACTTG GGCTGAATCC WTACCTTGGC ATCCTGGTGA ACAGCAAGTT  335
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          TTCTCACTTG GGCTGAATCC CTACCCTGGC ATCCTGGTGA ACAGCAAGTT  422
SR-6          TTCTCACTTG GGCTGAATCC CTACCCTGGC ATCCTGGTGA ACAGCAAGTT  772
C-fms Tag     TTCTCACTTG GGCTGAATCC CTACCCTGGC ATCCTGGTGA ACAGCAAGTT 2894
Consensus     TTCTCACTTG GGCTGAATCC WTACCYTGGC ATCCTGGTGA ACAGCAAGTT 2900


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           CTATAAACTG GTGAAGGATG GATACCAAAT GGCCCAGCCT GCATTTGCCC  385
SF-5          ---------- ---------- ---------- ---------- ----------
SF-4          CTATAAACTG GTGAAGGATG GATACCAAAT GGCCCAGCCT GCATTTGCCC  472
SR-6          CTATAAACTG GTGAAGGATG GATACCAAAT GGCCCAGCCT GCATTTGCCC  822
C-fms Tag     CTATAAACTG GTGAAGGATG GATACCAAAT GGCCCAGCCT GCATTTGCCC 2944
Consensus     CTATAAACTG GTGAAGGATG GATACCAAAT GGCCCAGCCT GCATTTGCCC 2950


SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
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SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           CAAAGAATAT ATACAGCATC ATGCAGGCCT GCTGGGCCTT GGAGCCCACC  435
SF-5          ---------- ---CAGCATC ATGCAAGNCT GCTGGGCCTT GGAGCCCACC   37
SF-4          CAAAGAATAT ATACAGCATC ATGCANGCCT GCTGGGCCTT GGAGCCCACC  522
SR-6          C---GAATAT ATACAG---- ---------- ---------- ----------  835
C-fms Tag     CAAAGAATAT ATACAGCATC ATGCAGGCCT GCTGGGCCTT GGAGCCCACC 2994
Consensus     CAAAGAATAT ATACAGCATC ATGCANGNCT GCTGGGCCTT GGAGCCCACC 3000

SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           CACAGACCCA CCTTCCAGCA GATCTGCTCC TTCCTTCAGG AGCAGGCCCA  485
SF-5          CACAGACCCA CCTTCCAGCA GATCTGCTCC TTCCTTCAGG AGCAGGCCCA   87
SF-4          CACAGACCCA CCTTCCAGCA GATCTGCTCC TTCCTTCANG AGCAAGCCCA  572
SR-6          ---------- ---------- ---------- ---------- ----------  835
C-fms Tag     CACAGACCCA CCTTCCAGCA GATCTGCTCC TTCCTTCAGG AGCAGGCCCA 3044
Consensus     CACAGACCCA CCTTCCAGCA GATCTGCTCC TTCCTTCANG AGCARGCCCA 3050

SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           AGAGGACAGG AGAGAGCGGG ACTATACCAA TCTGCCGAGC AGCAGCAGAA  535
SF-5          AGAGGACAGG AGAGAGCGGG ACTATACCAA TCTGCCGAGC AGCAGCAGAA  137
SF-4          AGAGGACAGG AGANAGCGGG ACTATAC-AA TCTGCCGAGC AGCAACAAAA  621
SR-6          ---------- ---------- ---------- ---------- ----------  835
C-fms Tag     AGAGGACAGG AGAGAGCGGG ACTATACCAA TCTGCCGAGC AGCAGCAGAA 3094
Consensus     AGAGGACAGG AGANAGCGGG ACTATACCAA TCTGCCGAGC AGCARCARAA 3100

SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           GCGGTGGCAG CGGCAGCAGC AGCAGTGAGC TGGAGGAGGA GAGCTCTAGT  585
SF-5          GCGGTGGCAG CGGCAGCAGC AGCAGTGAGC TGGAGGAGGA GAGCTCTAGT  187
SF-4          GCGGTGGNAG CG-NAACAAC AACAGTGAGC TGGAGGAGGA GA-CTCTA-T  668
SR-6          ---------- ---------- ---------- ---------- ----------  835
C-fms Tag     GCGGTGGCAG CGGCAGCAGC AGCAGTGAGC TGGAGGAGGA GAGCTCTAGT 3144
Consensus     GCGGTGGNAG CGGNARCARC ARCAGTGAGC TGGAGGAGGA GAGCTCTAGT 3150

SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
SR-5          ---------- ---------- ---------- ---------- ----------  817
SF-2          ---------- ---------- ---------- ---------- ----------  847
SR-4          ---------- ---------- ---------- ---------- ----------  780
SF-3          ---------- ---------- ---------- ---------- ----------  720
SR-3          ---------- ---------- ---------- ---------- ----------  754
SF-1          ---------- ---------- ---------- ---------- ----------  776
BGH           GAGCACCTGA CCTGCTGCGA GCAAGGGGAT ATCGCCCAGC CCTTGCTGCA  635
SF-5          GAGCACCTGA CCTGCTGCGA GCAAGGGGAT ATCGCCCAGC CCTTGCTGCA  237
SF-4          GAACACCTGA CCTGCTGC-A ACAAGGGGAT ATCGCC-AAC CCTTGCTGNA  716
SR-6          ---------- ---------- ---------- ---------- ----------  835
C-fms Tag     GAGCACCTGA CCTGCTGCGA GCAAGGGGAT ATCGCCCAGC CCTTGCTGCA 3194
Consensus     GARCACCTGA CCTGCTGCGA RCAAGGGGAT ATCGCCCARC CCTTGCTGNA 3200

SR-1          ---------- ---------- ---------- ---------- ----------  348
T7            ---------- ---------- ---------- ---------- ----------  833
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SR-5            ---------- ---------- ---------- ---------- ---------- 817
SF-2            ---------- ---------- ---------- ---------- ---------- 847
SR-4            ---------- ---------- ---------- ---------- ---------- 780
SF-3            ---------- ---------- ---------- ---------- ---------- 720
SR-3            ---------- ---------- ---------- ---------- ---------- 754
SF-1            ---------- ---------- ---------- ---------- ---------- 776
BGH             GCCCAACAAC TATCAGTTCT GCAAGCTTCG AAGCCGAATT CTGCAGATAT 685
SF-5            GCCCAACAAC TATCAGTTCT GCAAGCTTCG AAGCCGAATT CTGCAGATAT 287
SF-4            G-CCAACAAC TATCAATTCT GCAAGCTTC- AAACCGAATT CT-CAAATAT 763
SR-6            ---------- ---------- ---------- ---------- ---------- 835
C-fms Tag       GCCCAACAAC TATCAGTTCT GCAAGCTTCG AAGCCGAATT CTGCAGATAT 3244
Consensus       GCCCAACAAC TATCARTTCT GCAAGCTTCG AARCCGAATT CTGCARATAT 3250

SR-1            ---------- ---------- ---------- ---------- ---------- 348
T7              ---------- ---------- ---------- ---------- ---------- 833
SR-5            ---------- ---------- ---------- ---------- ---------- 817
SF-2            ---------- ---------- ---------- ---------- ---------- 847
SR-4            ---------- ---------- ---------- ---------- ---------- 780
SF-3            ---------- ---------- ---------- ---------- ---------- 720
SR-3            ---------- ---------- ---------- ---------- ---------- 754
SF-1            ---------- ---------- ---------- ---------- ---------- 776
BGH             CCATCACACT GGCGGCCGCT CGAGGTCACC CATTCGAACA AAAACTCATC 735
SF-5            CCATCACACT GGCGGCCGCT CGAGGTCACC CATTCGAACA AAAACTCATC 337
SF-4            -CATCAAACT GGNGGGCGCT CCANGTCA-C CANTC-AAC- AAAACTCATC 809
SR-6            ---------- ---------- ---------- ---------- ---------- 835
C-fms Tag       CCATCACACT GGCGGCCGCT CGAGGTCACC CATTCGAACA AAAACTCATC 3294
Consensus       CCATCAMACT GGNGGSCGCT CSANGTCACC CANTCGAACA AAAACTCATC 3300

SR-1            ---------- ---------- ---------- ---------- ---------- 348
T7              ---------- ---------- ---------- ---------- ---------- 833
SR-5            ---------- ---------- ---------- ---------- ---------- 817
SF-2            ---------- ---------- ---------- ---------- ---------- 847
SR-4            ---------- ---------- ---------- ---------- ---------- 780
SF-3            ---------- ---------- ---------- ---------- ---------- 720
SR-3            ---------- ---------- ---------- ---------- ---------- 754
SF-1            ---------- ---------- ---------- ---------- ---------- 776
BGH             TCAGTTGAGG ATCTGAATAG NCN-ACCGGT CAT------- ---------- 767
SF-5            TCAGAAGAGG ATCTGAATAT GCATACCGGT CATCATCACC ATCACCATTG 387
SF-4            -AAAAAGAGG GTTTG-ATAT GCATACCGGT CA-------- ---------- 839
SR-6            ---------- ---------- ---------- ---------- ---------- 835
C-fms Tag       TCAGAAGAGG ATCTGAATAT GCATACCGGT CATCATCACC ATCACCATTG 3344
Consensus       TSARNNGAGG RTYTGAATAK NCNTACCGGT CATCATCACC ATCACCATTG 3350

SR-1            ---------- -          348
T7              ---------- -          833
SR-5            ---------- -          817
SF-2            ---------- -          847
SR-4            ---------- -          780
SF-3            ---------- -          720
SR-3            ---------- -          754
SF-1            ---------- -          776
BGH             ---------- -          767
SF-5            AGTTTAAACC C          398
SF-4            ---------- -          839
SR-6            ---------- -          835
C-fms Tag       AGTTTAAACC C          3355
Consensus       AGTTTAAACC C          33
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Sequence analysis of mutations at Leu^{301}

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C-fms CTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     1142

WT-1R CTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     361
WT-1F CTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     380

MT-5F CTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     423
MT-5R TTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     395

MT-6F CTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     424
MT-6R TTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     391

MT-7F CTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     412
MT-7R TTCGATCAAG TAGATTTCCA ACATGCCGGC AACTACTCCT GCGTGGCCAG     396
      ********** ********** ********** ********** **********

C-fms CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     1192

WT-1R CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     411
WT-1F CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     430

MT-5F CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     473
MT-5R CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     445

MT-6F CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     474
MT-6R CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     441

MT-7R CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     462
MT-7F CAACGTGCAG GGCAAGCACT CCACCTCCAT GTTCTTCCGG GTGGTAGAGA     446
      ********** ********** ********** ********** **********

C-fms GTGCCTACTT GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     1242

WT-1R GTGCCTACTT GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     461
WT-1F GTGCCTACTT GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     480

MT-5F GTGCCTACTC GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     523
MT-5R GTGCCTACTC GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     495

MT-6F GTGCCTACTC GAACTTGAGC TCTGAGCAGA ACCTCATCCA AGAGGTGACC     524
MT-6R GTGCCTACTC GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     491

MT-7F GTGCCTACTC GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     512
MT-7R GTGCCTACTC GAACTTGAGC TCTGAGCAGA ACCTCATCCA GGAGGTGACC     496
      ********* ********** ********** ********** **********

C-fms GTGGGGGAGG GGCTCAACCT CAAAGTCATG GTGGAGGCCT ACCCAGGCCT     1292

WT-1R GTGGGGGAGG GGCTCAACCT CAAAGTCATG GTGGAGGC-T ACCCAGGCCT     511
WT-1F GTGGGGGAGG GGCTCAACCT CAAAGTCATG GTGGAGGC-T ACCCAGGCCT     530

MT-5F GTGGGGGAAG GGCTCAACCT CAAAGTCATG GTGGAGGCCT ACCCAAGCCT     573
MT-5R GTGGGGGAGG GGCTCAACCT CAAAGTCATG GTGGAGGCCT ACCCAGGCCT     545

MT-6F GTGGGGGAAG GGCTCAACCT CAAAGTCATG GTGGAGGC-T AACCAGGCCT     573
MT-6R GTGGGGGAGG GGCTCAACCT CAAAGTCATG GTGGAGGCCT ACCCAGGCCT     541

MT-7F GTGGGGGAAG GGCTCAACCT CAAAGTCATG GTGGAGGCCT ACCCAGGCCT     562
MT-7R GTGGGGGAGG GGCTCAACCT CAAAGTCATG GTGGAGGCCT ACCCAGGCCT     546
      ******** * ********** ********** ******** * **********
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Multiple alignment of Tyr^{708} and Tyr^{723} mutants

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C-fms CACGGAGTA- CTGTTGCTAT GGCGACCTG- CTCAACTTTC TGCGAAGGAA 2333

MT-1F CACGGAGTA- CTGTTGCTAT GGCGACCTG- CTCAACTTTC TGCGAAGGAA 300
MT-1R CACGGAGTA- CTGTTGCTAT GGCGACNTG-- TCAACTTTT TGCGAAGGAA 176

MT-2F CACGGAGTA- CTGTTGCTAT GGCGACCTTG CTCAACTTTC TGCGAAGGAA 342

MT-3R CACGGAGTAA TTGTTGNNAT GGCGACTTG- CTCAACTTTT TGCGAAGGAA 156
MT-3F CACGGAGTA- CTGTTGCTAT GGCGACC-TG CTCAACTTTC TGCGAAGGAA 359

MT-4R CACGGAGTA- CTGTTGCTAT GGCGACC-TG CTCAACTTTC TGCGAAGGAA 360
MT-4F AACGGAGTAN TTGTTGTTAT GGCGRCCTKG -TAAACTTTT TGNGAAGGAA 126

MT-5R NAGGGAGTAC TTGTTGNTAT GGCGACCTTG CTCAACTTTC TGCGAAGGAA 159
MT-5F CACGGAGTA- CTGTTGCTAT GGCGACC-TG CTCAACTTTC TGCGAAGGAA 359

MT-6R CACGGAGTA- CTGTTGCTAT GGCGACC-TG CTCAACTTTC TGCGAAGGAA 354


C-fms GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 2380

MT-1F GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 350
MT-1R GG-CTGAGGC CA-TGCTGG- ACCCAGCTTG AGCCCC-GGC CAGGACCCCG 226

MT-2F GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 392

MT-3R GG-CTGAGCC CA-TGTTGGG ACCCAGCTTG AGCCCC-GGC CAGGACCCGG 203
MT-3F GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 406

MT-4R GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 407
MT-4F GGGTTGAGCC CATTGCT-GG ACCCAGCTTG AGCCCCGG-C CAGGACCCCG 174

MT-5R GG-CTGAGCC CAT-GCTGGG ACCCAGCTTG AGCCCCGGGC CAGGACCCCG 207
MT-5F GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 406

MT-6R GG-CTGAGGC CA-TGCTGGG ACCCAGCCTG AGCCCC-GGC CAGGACCCCG 401


C-fms AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATATGTCCG 2429

MT-1F AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATATGTCCG 400
MT-1R AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATATGTCCG 276

MT-2F AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATTTGTCCG 442

MT-3R AGGGAGGCGT CGACTATTAA GAACATCCAC CTCGAGAAGA AATATGTCCG 253
MT-3F AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATATGTCCG 455

MT-4R AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATTTGTCCG 456
MT-4F AGGGAGGCGT CGACTAT-AA GAACATCCAC CTCGAGAAGA AATTTGTCCG 223

MT-5R AGGGAGGCGT CGACTAT-AG GAACATCCAC TTCGAGAAGA AATTTGTCCG 256
MT-5F AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATTTGTCCG 455

MT-6R AGGGAGGCGT CGACTA-TAA GAACATCCAC CTCGAGAAGA AATATGTCCG 450
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C-fms CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTAT GTGGAGATGA     2450

MT-1F CAGGGACAGT GGCTTCTCCA GCCANGGTGT GGACACCTAT GTGGAGATGA     450
MT-1R CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTAT GTGGAGATGA     326

MT-2F CAGGGACA-T GGCTTCTCCA GCCAGGGTGT GGACACCTAT GTGGNGATGA     492

MT-3R CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTTT GTGGAGATGA     274
MT-3F CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTTT GTGGAGATGA     505

MT-4R CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTTT GTGGAGATGA     506
MT-4F CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTTT GTGGAGATGA     273

MT-5R CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTAT GTGGAGATGA     277
MT-5F CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTAT GTGGAGATGA     505

MT-6R CAGGGACAGT GGCTTCTCCA GCCAGGGTGT GGACACCTTT GTGGAGATGA     500
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C-fms GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     2497

MT-1F GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     500

MT-1R GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     376

MT-2F GGC-TGTCTC CACTTCTTCA AATGA-TCCT TCTCTGAACA AGACCTGGAC     542

MT-3R GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     321
MT-3F GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     523

MT-4R GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     323
MT-4F GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     556

MT-5R GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     523
MT-5F GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     324

MT-6R GGCCTGTCTC CACTTCTTCA AATGACTCCT TCTCTGAGCA AGACCTGGAC     518
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C-fms AAGGAGGATG GACGGCCC     2521

MT-1F AAGGAGGATG GACGG---     524
MT-1R AAGGAGGATG GACGGCCC     426

MT-2F AAGGAGGATG GACGGCCC     592

MT-3R AAGGAGGATG GACGGCCC     345
MT-3F AAGGAGGATG GA------     541

MT-4R AAGGAGGATG GACGGCCC     347
MT-4F AAGGAGGATG GACGGCCC     580

MT-5R AAGGAGGATG GACGGCCC     348
MT-5F AAGGAGGATG GA------     541

MT-6R AAGGAGGATG GA------     536
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Alignment of translated MT-1 with the published human c-fms sequence

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C-fms Tagged      GFRKGRQSVQ KRESTKGEGG EERRGRGRGR EEAEGTAARL KGEEEDQPKE   50
Published c-fms   ---------- ---------- ---------- ---------- ----------

Consensus         .......... .......... .......... .......... ..........   50

C-fms Tagged      EEEENKTNSQ CRGEERVSSV PIPAELVAES SVPWAPCSPA PACHFPTEAM   100
Published c-fms   ---------- ---------- ---------- ---------- ---------M   1

Consensus         .......... .......... .......... .......... .........M   100

C-fms Tagged      GPGVLLLLLV ATAWHGQGIP VIEPSVPELV VKPGATVTLR CVGNGSVEWD   150
Published c-fms   GPGVLLLLLV ATAWHGQGIP VIEPSVPELV VKPGATVTLR CVGNGSVEWD   51

Consensus         GPGVLLLLLV ATAWHGQGIP VIEPSVPELV VKPGATVTLR CVGNGSVEWD   150

C-fms Tagged      GPASPHWTLY SDGSSSILST NNATFQNTGT YRCTEPGDPL GGSAAIHLYV   200
Published c-fms   GPASPHWTLY SDGSSSILST NNATFQNTGT YRCTEPGDPL GGSAAIHLYV   101

Consensus         GPASPHWTLY SDGSSSILST NNATFQNTGT YRCTEPGDPL GGSAAIHLYV   200

C-fms Tagged      KDPARPWNVL AQEVVVFEDQ DALLPCLLTD PVLEAGVSLV RVRGRPLMRH   250
Published c-fms   KDPARPWNVL AQEVVVFEDQ DALLPCLLTD PVLEAGVSLV RVRGRPLMRH   151

Consensus         KDPARPWNVL AQEVVVFEDQ DALLPCLLTD PVLEAGVSLV RVRGRPLMRH   250

C-fms Tagged      TNYSFSPWHG FTIHRAKFIQ SQDYQCSALM GGRKVMSISI RLKVQKVIPG   300
Published c-fms   TNYSFSPWHG FTIHRAKFIQ SQDYQCSALM GGRKVMSISI RLKVQKVIPG   201

Consensus         TNYSFSPWHG FTIHRAKFIQ SQDYQCSALM GGRKVMSISI RLKVQKVIPG   300

C-fms Tagged      PPALTLVPAE LVRIRGEAAQ IVCSASSVDV NFDVFLQHNN TKLAIPQQSD   350
Published c-fms   PPALTLVPAE LVRIRGEAAQ IVCSASSVDV NFDVFLQHNN TKLAIPQQSD   251

Consensus         PPALTLVPAE LVRIRGEAAQ IVCSASSVDV NFDVFLQHNN TKLAIPQQSD   350

C-fms Tagged      FHNNRYQKVL TLNLDQVDFQ HAGNYSCVAS NVQGKHSTSM FFRVVESAYL   400
Published c-fms   FHNNRYQKVL TLNLDQVDFQ HAGNYSCVAS NVQGKHSTSM FFRVVESAYL   301

Consensus         FHNNRYQKVL TLNLDQVDFQ HAGNYSCVAS NVQGKHSTSM FFRVVESAYL   400

C-fms Tagged      NLSSEQNLIQ EVTVGEGLNL KVMVEAYPGL QGFNWTYLGP FSDHQPEPKL   450
Published c-fms   NLSSEQNLIQ EVTVGEGLNL KVMVEAYPGL QGFNWTYLGP FSDHQPEPKL   351

Consensus         NLSSEQNLIQ EVTVGEGLNL KVMVEAYPGL QGFNWTYLGP FSDHQPEPKL   450

C-fms Tagged      ANATTKDTYR HTFTLSLPRL KPSEAGRYSF LARNPGGWRA LTFELTLRYP   500
Published c-fms   ANATTKDTYR HTFTLSLPRL KPSEAGRYSF LARNPGGWRA LTFELTLRYP   401

Consensus         ANATTKDTYR HTFTLSLPRL KPSEAGRYSF LARNPGGWRA LTFELTLRYP   500

C-fms Tagged      PEVSVIWTFI NGSGTLLCAA SGYPQPNVTW LQCSGHTDRC DEAQVLQVWD   550
Published c-fms   PEVSVIWTFI NGSGTLLCAA SGYPQPNVTW LQCSGHTDRC DEAQVLQVWD   451

Consensus         PEVSVIWTFI NGSGTLLCAA SGYPQPNVTW LQCSGHTDRC DEAQVLQVWD   550

C-fms Tagged      DPYPEVLSQE PFHKVTVQSL LTVETLEHNQ TYECRAHNSV GSGSWAFIPI   600
Published c-fms   DPYPEVLSQE PFHKVTVQSL LTVETLEHNQ TYECRAHNSV GSGSWAFIPI   501

Consensus         DPYPEVLSQE PFHKVTVQSL LTVETLEHNQ TYECRAHNSV GSGSWAFIPI   600

C-fms Tagged      SAGAHTHPPD EFLFTPVVVA CMSIMALLLL LLLLLLYKYK QKPKYQVRWK   650
Published c-fms   SAGAHTHPPD EFLFTPVVVA CMSIMALLLL LLLLLLYKYK QKPKYQVRWK   551

Consensus         SAGAHTHPPD EFLFTPVVVA CMSIMALLLL LLLLLLYKYK QKPKYQVRWK   650
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C-fms Tagged        IIESYEGNSY TFIDPTQLPY NEKWEFPRNN LQFGKTLGAG AFGKVVEATA    700
Published c-fms     IIESYEGNSY TFIDPTQLPY NEKWEFPRNN LQFGKTLGAG AFGKVVEATA    601

Consensus           IIESYEGNSY TFIDPTQLPY NEKWEFPRNN LQFGKTLGAG AFGKVVEATA    700


C-fms Tagged        FGLGKEDAVL KVAVKMLKST AHADEKEALM SELKIMSHLG QHENIVNLLG    750
Published c-fms     FGLGKEDAVL KVAVKMLKST AHADEKEALM SELKIMSHLG QHENIVNLLG    651

Consensus           FGLGKEDAVL KVAVKMLKST AHADEKEALM SELKIMSHLG QHENIVNLLG    750


C-fms Tagged        ACTHGGPVLV ITEYCCYGDL LNFLRRKAEA MLGPSLSPGQ DPEGGVDYKN    800
Published c-fms     ACTHGGPVLV ITEYCCYGDL LNFLRRKAEA MLGPSLSPGQ DPEGGVDYKN    701

Consensus           ACTHGGPVLV ITEYCCYGDL LNFLRRKAEA MLGPSLSPGQ DPEGGVDYKN    800


C-fms Tagged        IHLEKKYVRR DSGFSSQGVD TYVEMRPVST SSNDSFSEQD LDKEDGRPLE    850
Published c-fms     IHLEKKYVRR DSGFSSQGVD TYVEMRPVST SSNDSFSEQD LDKEDGRPLE    751

Consensus           IHLEKKYVRR DSGFSSQGVD TYVEMRPVST SSNDSFSEQD LDKEDGRPLE    850


C-fms Tagged        LRDLLHFSSQ VAQGMAFLAS KNCIHRDVAA RNVLLTNGHV AKIGDFGLAR    900
Published c-fms     LRDLLHFSSQ VAQGMAFLAS KNCIHRDVAA RNVLLTNGHV AKIGDFGLAR    801

Consensus           LRDLLHFSSQ VAQGMAFLAS KNCIHRDVAA RNVLLTNGHV AKIGDFGLAR    900


C-fms Tagged        DIMNDSNYIV KGNARLPVKW MAPESIFDCV YTVQSDVWSY GILLWEIFSL    950
Published c-fms     DIMNDSNYIV KGNARLPVKW MAPESIFDCV YTVQSDVWSY GILLWEIFSL    851

Consensus           DIMNDSNYIV KGNARLPVKW MAPESIFDCV YTVQSDVWSY GILLWEIFSL    950


C-fms Tagged        GLNPYPGILV NSKFYKLVKD GYQMAQPAFA PKNIYSIMQA CWALEPTHRP   1000
Published c-fms     GLNPYPGILV NSKFYKLVKD GYQMAQPAFA PKNIYSIMQA CWALEPTHRP    901

Consensus           GLNPYPGILV NSKFYKLVKD GYQMAQPAFA PKNIYSIMQA CWALEPTHRP   1000


C-fms Tagged        TFQQICSFLQ EQAQEDRRER DYTNLPSSSR SGGSGSSSSE LEEESSSEHL   1050
Published c-fms     TFQQICSFLQ EQAQEDRRER DYTNLPSSSR SGGSGSSSSE LEEESSSEHL    951

Consensus           TFQQICSFLQ EQAQEDRRER DYTNLPSSSR SGGSGSSSSE LEEESSSEHL   1050


C-fms Tagged        TCCEQGDIAQ PLLQPNNYQF CKLRSRILQI SITLAAARGH PFEQKLISEE   1100
Published c-fms     TCCEQGDIAQ PLLQPNNYQF C*-------- ---------- ----------    972

Consensus           TCCEQGDIAQ PLLQPNNYQF C......... .......... ..........   1100


C-fms Tagged        DLNMHTGHHH HHH 1113
Published c-fms     ---------- --- 972

Consensus           .......... ... 1113
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Diagram of epitope tags present in MT-1 construct protein translation.

SEQUENCE: 1014aa 112837 predicted MW

MGPGVLLLLLVATAWHGQGIPVIEPSVPELVVKPGATVTLRCVGNGSVEW
DGPASPHWTLYSDGSSSILSTNNATFQNTGTYRCTEPGDPLGGSAAIHLY
VKDPARPWNVLAQEVVVFEDQDALLPCLLTDPVLEAGVSLVRVRGRPLMR
HTNYSFSPWHGFTIHRAKFIQSQDYQCSALMGGRKVMSISIRLKVQKVIP
GPPALTLVPAELVRIRGEAAQIVCSASSVDVNFDVFLQHNNTKLAIPQQS
DFHNNRYQKVLTLNLDQVDFQHAGNYSCVASNVQGKHSTSMFFRVVESAY
LNLSSEQNLIQEVTVGEGLNLKVMVEAYPGLQGFNWTYLGPFSDHQPEPK
LANATTKDTYRHTFTLSLPRLKPSEAGRYSFLARNPGGWRALTFELTLRY
PPEVSVIWTFINCSCTLLCAASGYPQPNVTWLQCSGHTDRCDEAQVLQVW
DDPYPEVLSQEPFHKVTVQSLLTVETLEHNQTYECRAHNSVGSGSWAFIP
ISAGAHTHPPDEFLFTPVVVACMSIMALLLLLLLLLLYKYKQKPKYQVRW
KIIESYEGNSYTFIDPTQLPYNEKWEFPRNNLQFGKTLGAGAFGKVVEAT
AFGLGKEDAVLKVAVKMLKSTAHADEKEALMSELKIMSHLGQHENIVNLL
GACTHGGPVLVITEYCCYGDLLNFLRRKAEAMLGPSLSPGQDPEGGVDYK
NIHLEKKYVRRDSGFSSQGVDTYVEMRPVSTSSNDSFSEQDLDKEDGRPL
ELRDLLHFSSQVAQGMAFLASKNCIHRDVAARNVLLTNGHVAKIGDFGLA
RDIMNDSNYIVKGNARLPVKWMAPESIFDCVYTVQSDVWSYGILLWEIFS
LGLNPYPGILVNSKFYKLVKDGYQMAQPAFAPKNIYSIMQACWALEPTHR
PTFQQICSFLQEQAQEDRRERDYTNLPSSSRSGGSGSSSSELEEESSSEH
LTCCEQGDIAQPLLQPNNYQFCKLRSRILQISITLAAARGHPFEQKLISE
EDLNMHTGHHHHHH

C-fms

Linker

myc-epitope

His-epitope

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